THE ROLE OF TELOMERE LENGTH IN TREE SWALLOW BEHAVIOR AND LIFE HISTORY

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THE ROLE OF TELOMERE LENGTH IN TREE SWALLOW BEHAVIOR AND LIFE HISTORY

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Telomeres are the protective caps of chromosomes, and variation in telomere length (TL) in many species is correlated with mortality, disease, and reproduction. Thus, TL may be a reasonable measure of individual quality, and it is important to better understand the causes of TL variation and its consequences for behavior and life history. To study the role TL plays in an individuals behavior and life history, we enlarged broods in a wild population of Tree Swallows (*Tachycineta bicolor*) and followed both chicks and adults.

First we used this manipulation to study the determinants of early-life TL. Early-life TL was highly heritable and the heritable contribution from the female was higher than from males. The manipulation only weakly shortened chick TL, and extra-pair status of the young did not affect their TL. Extra-pair young had a higher probability of fledging, but only in enlarged broods.

We then looked at the adults to study the consequences of TL variation. We found that adult males had longer TL than did females, but that parental TL did not correlate with any proxy of fitness measured. Parental TL did not predict the response of parents to brood enlargement, but we *did* find assortative mating for TL and evidence that the TL of the *mate* is an important factor in an individual's reproductive-investment decisions.

Together, these results suggest that the role TL plays in life history, and its use

as a proxy for quality, are context-dependent. In chicks, the stage in development at which we measure TL will affect how well it functions as a measure of quality. In adults, we might see different patterns of correlation between TL and fitness depending on the life-history particulars of the species, if it is short or long-lived, how susceptible it is to adverse weather conditions, etc. Lastly, adults might be responding adaptively to their own quality and TL, as well as their mates TL, in complex ways, masking direct correlations between TL and fitness. Future studies should explicitly consider these subtle variations in context when they are testing both the determinants and consequences of TL variation.

BIOGRAPHICAL SKETCH

Amos Belmaker finished his undergraduate B.Sc., *cum laude*, at the Faculty of Life-Sciences at Tel-Aviv University in 2005. He completed his undergraduate honors thesis under the supervision of Prof. Arnon Lotem studying the response of House Sparrow chicks to artificial playback sounds.

Amos then went on to complete an M.Sc., *magna cum laude*, in the field of Ecology at the Department of Zoology in Tel-Aviv University, also under the supervision of Prof. Arnon Lotem. For his masters, Amos studied adult learning in the context of the producer-scrounger game in House Sparrows.

Before continuing on for his Ph.D., Amos joined the 'Golondrinas de las Americas' project led by Prof. David Winkler, and worked for two seasons studying *Tachycineta* swallows in Tierra del Fuego, Argentina, and Mono Lake, California.

Amos started his Ph.D at Cornell University in August 2009. He was supervised by Prof. David. Winkler in the Department of Ecology and Evolutionary Biology and the Cornell Laboratory of Ornithology.

This dissertation is dedicated to my family. To my wife Dennise, who agreed to leave everything behind and come with me to a different country for six years — I couldn't have done it without her love and support. To my fitness units, Ilan and Tamar, who always make me smile.

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CHAPTER 1

THE ENVIRONMENTAL AND GENETIC DETERMINANTS OF CHICK TELOMERE LENGTH IN TREE SWALLOWS (*Tachycineta bicolor*)

Abstract

The period of early growth has been identified as critical in its effect on performance and fitness later in life. However, we still know little about the mechanisms that mediate these effects of early growth. One potential mechanism by which development can exact an effect on fitness is through telomere shortening. Telomeres are the long sequences of DNA that protect the ends of chromosomes. Short telomeres are associated with poor health, low quality and lower survival. Given that telomere length (TL) is associated with fitness, natural selection should favor individuals that manage to retain longer telomeres. However, the extent to which this is possible depends on the heritability of TL on the one hand and on the strength of environmental effects on TL on the other. Here we enlarged broods of Tree Swallows to test the effects of demanding early-growth conditions on TL and cross-fostered chicks to estimate heritable genetic influences on TL. We found that TL is highly heritable, with heritable influences stronger from mothers than from fathers. In addition, maternal telomere length is more closely related to that of her daughters than that of her sons. The experimental manipulation had a weak effect on chick TL, possibly because chicks were sampled too early in development. These results suggest that early in life TL is determined more by heritable factors than environmental ones, and that stress probably plays a bigger role in TL variation as the young mature.

Introduction

Within the development of individual organisms, the period of early growth has been identified as critical to performance later in life and fitness (Lindström 1999; Watson et al. 2015). However, we know little about the mechanisms that mediate the effects of early growth on subsequent performance (Monaghan and Haussmann 2006). One potential mechanism by which development can affect fitness is through its effects on telomere shortening (Heidinger et al. 2012).

Telomeres are the long repetitive, non-coding sequences of DNA that cap and protect the ends of eukaryote chromosomes (Blackburn 2000). As the chromosomes shorten with each replication, there is a danger that important genetic information will be lost (Levy et al. 1992). During each replicative shortening of the chromosome, telomeres protect the DNA by being shortened instead of coding and structural DNA sequences farther from the chromosome ends (Levy et al. 1992). In addition, telomeres prevent the DNA repair mechanism from falsely identifying chromosome ends as double-stranded breaks (Nugent et al. 1998). When telomere length (TL) shortens beneath a certain threshold, the cell becomes senescent, starting a cascade that can ultimately lead to cell death, reduced organ function and eventual death of the individual (Campisi 2005). Because short telomeres trigger this deleterious cascade, they are associated with poor health (Bojesen 2013) and lower survival (Haussmann and Marchetto 2010), and are used as a proxy for low quality in many species, with short-telomered individuals having lower reproductive success (i.e., Le Vaillant et al. 2015; but see Bauch et al. 2013).

The large body of evidence showing environmental effects on telomere length strengthens this use of telomere length as a proxy for individual quality (Bauch et al.

2013; Le Vaillant et al. 2015). In addition to the per-replication shortening of TL (Levy et al. 1992), stress (Epel et al. 2004) and oxidative damage (Saretzki and Von Zglinicki 2002) act to shorten telomeres as well. In fact, many physiological factors are either directly or indirectly associated with telomere attrition in a myriad of pathways (Haussmann and Marchetto 2010) making TL a complex trait influenced by, and affecting, the expression of many different genes (Gatbonton et al. 2006). Both the rapid division of cells in early life and the sensitivity to disturbances during that period accelerate TL shortening during early development (Baerlocher et al. 2007; Hall et al. 2004; Nettle et al. 2015; Salomons et al. 2009). Indeed, most telomere shortening happens early in life (Haussmann and Marchetto 2010), and TL shortening is connected to rapid catch-up growth (i.e., Geiger et al. 2012). Thus, environmental factors during the sensitive period of early growth, through their effect on telomere shortening, can have a profound effect on an individual's performance later in life (Nettle et al. 2015). Despite the fact that telomere erosion is deleterious in all ages, early-life TL seems to be a better predictor of fitness than late-life TL (Heidinger et al. 2012). Thus, studying the causes of variation in early-life TL will help us understand how TL can mediate the effects of early growth environment on performance and fitness (Watson et al. 2015). Regardless of how strong these environmental effects are, they will necessarily interact with the heritable influences on TL variation, so estimating the heritability of TL early in life is crucial if we are to understand how early-life TL mediates the effects of development on performance later in life.

The heritability of TL (the proportion of variation in TL that is due to additive genetic factors or h^2) has been studied mainly in humans, with estimates ranging from 0.30 to 1.28 (Atema et al. 2015, Note that h^2 values greater than one are obtained in regressions of the offspring trait value on that of a single parent and multiplying by two). In contrast, in other vertebrates TL heritability estimates are rare, with values ranging anywhere from 0.09 to 1.23 depending on the species (for a full list see Atema et al. 2015). The individual's

sex seems also to play a key role in telomere dynamics and inheritance. In several cases TL has been found to differ between sexes (reviewed in Barrett and Richardson 2011), and the correlation between parental TL and that of the offspring has been found to be stronger for one sex than the other (Nordfjäll et al. 2009; Reichert et al. 2015a). While it has been suggested that inheritance is stronger from the parent of the heterogametic sex, there are examples where this is not the case (i.e., Broer et al. 2013).

When TL heritability is measured it is important to remember that TL is a complex trait with many associated genes controlling it (Codd et al. 2010; Gatbonton et al. 2006) and that variation in TL is determined by both heritable and environmental components. The TL of an individual starts out in the zygote with the actual telomeric sequence that is inherited from one or both parents (De Meyer et al. 2014; Graakjaer et al. 2004), and the TL passed on to the offspring depends on telomere dynamics in the germline of the parents. However, stress, oxidative and otherwise (Haussmann and Marchetto 2010), can reduce TL, and telomerase, the enzyme that adds telomeric repeats to the end of the chromosome, can increase it. Each of these effects involves genes and gene products that are part of the complicated TL causative web (Haussmann and Marchetto 2010; Simons 2015): the TL of parents and offspring can be similar through shared telomeric sequences passed on at the zygote stage (De Meyer et al. 2014), or due to the actions of genes associated with TL (Haussmann and Marchetto 2010). These two different modes in TL inheritance can act independently from each other (De Meyer et al. 2014), and, depending on the life stage at which we measure the offspring, we might be measuring the heritability of different traits—either the inheritance of telomere sequences or the inheritance of genes associated with TL.

TL heritability and environmental effects on early-life TL have been measured separately many times (see above). However, studies that estimate TL heritability in

the wild, while simultaneously directly manipulating the environment of developing offspring, are rare (Voillemot et al. 2012). To fill this gap and study the interaction of TL heritability and early-growth environment we cross-fostered Tree Swallow chicks in enlarged and control broods and measured TL of parents and offspring. Adults and chicks were genotyped and sexed, allowing us to look at sex effects on TL inheritance and to control for the high extra-pair paternity rate in Tree Swallows (Conrad et al. 2001). Studies in Tree Swallows have shown that adult TL shortens with age (Haussmann et al. 2003; but see Belmaker 2016, chapter 3) and that adult TL is associated with adult return rates (Haussmann et al. 2005; but see Belmaker 2016, chapter 3). In addition, telomerase is active in Tree Swallow chicks and not in adults (Haussmann et al. 2004; Haussmann et al. 2007), and longer-telomered adults raise lighter chicks (Ouyang et al. 2016). To our knowledge, there are no studies to look at telomere dynamics and heritability in the early-life of Tree Swallows.

This experimental design allows us to study both environmental and heritable influences on TL and their interaction, thus providing a richer view of the determinants of early-life TL. Because this is one of relatively few studies to combine an environmental manipulation with a heritability study (Voillemot et al. 2012), we hope it provides valuable perspective on findings conducted on early-life TL variation in laboratory settings (Reichert et al. 2015b).

Methods

Study system

During the breeding seasons of 2012-2014 we enlarged broods of Tree Swallows (*Tachycineta bicolor*) breeding in Harford, NY (42.44°N, 76.23°W). The Tree Swallow is a small, migratory, aerial insectivore, that has been used as a model system for studies of traits ranging from life history and behavior to physiology (Jones 2003). The study site is a cattle grazing ground with 130 nest boxes mounted on fence posts, ~1.5 m above the ground and at a distance of at least 20 m between boxes. Annual occupancy of the nest boxes is ~70 %, providing the opportunity for relatively large sample sizes. Nests were monitored daily once nest building was advanced to find the day the first egg was laid in each occupied box (clutch initiation date). Clutch completion was set as the day the last egg was laid. During incubation, boxes were monitored every third day to minimize interruption. Females were caught in the box after the seventh day of incubation to minimize the risk of abandonment. Males are harder to catch than females, and they were only caught after the manipulations described below were performed.

In the hand, every bird was measured for body mass, head-plus-bill length and wing length. For each individual we noted its age. Tree Swallow females show delayed plumage maturation, in which second year (SY) birds (first-time breeders) have a brown plumage, and older birds show the characteristic iridescent blue plumage (Hussell 1983). While this aging method is only accurate 95 % of the time (Hussell 1983) it still allows us to know the age of most female breeders with high accuracy. In addition, some individuals that fledged from our site returned to breed, and we can thus know their known age from

their unique band numbers. In cases where age was not determinable by these means, we noted the minimum age of each individual. It should be noted that using minimum-age as a predictor is conservative, as it tends to underestimate the effect of age. Lastly, a blood sample was taken from the brachial vein for telomere length analysis and genotyping. A minimum of 20 and a maximum of 150 µl was taken into a heparinized microcapillary tube. Half of the blood was put into lysis buffer for genotyping and was stored at room temperature. The other half was put into an empty 1.5 ml microcentrifuge tube and stored on ice until further processing in the lab. At the end of each day, telomere samples were spun down at 3500 rpm for 5 minutes, and the plasma was removed. One ml of NBS buffer (90 % new-born calf serum and 10 % DMSO) was added and mixed with the Red Blood Cells (RBCs). The samples were then frozen slowly and kept at −80 °C for storage until analysis.

Experimental manipulation

Once all chicks in a brood hatched, typically together on the same day but some times as much as two days apart, broods were paired with another brood of the same size, female age and hatch date. When exact female age was not known, we used minimum age to match the broods. We then randomly assigned one of the nests as a control nest and the other as an enlarged one. We first swapped 50 % of the broods between the matched nests. The hatching box of each chick was noted, and chicks were identified individually by clipping nails. We then added to the enlarged brood three chicks from a nest not participating in the experiment. These added chicks hatched on the same day as the manipulated chicks to ensure they were comparable in size. We did not include a reduced-sized brood treatment to ensure the largest sample possible of matched broods:

it is easier to find matches for two nests than for three. We enlarged each brood by ~50 %, as this treatment strength has already been shown to affect measurably the reproductive performance of Tree Swallow females (Ardia 2005). Final brood sizes for control broods averaged 5.07 ± 0.67 and 8.05 ± 0.86 for enlarged broods (mean±sd). Once the brood enlargement was done, we followed the breeding attempt to its conclusion. We measured the chicks for mass, head-plus-bill and wing length every four days until day 12. On day 12 the surviving chicks were measured, banded and a blood sample was taken for telomere measurement and genotyping. After day 12 the box was not opened to minimize the risk of premature fledging. To check when the chicks fledged we just peeked into the box. Fledging date was noted as the date the last chick fledged. After all surviving chicks fledged we noted the band number or marking of any dead chick left in the box. Any chicks that died before day 12 were genotyped but, as the TRF assay is sensitive to DNA degradation (Haussmann and Mauck 2008a), we could not get a TL estimate from those chicks.

Telomere length analysis

Telomeres were measured using the TRF assay (Kimura et al. 2010). Samples were thawed at 37 °C for 2 minutes and then spun down at 3500 rpm for 5 minutes. The supernatant was discarded. DNA was extracted from the remaining RBCs using a Gentra Purgene extraction kit for the extraction of high quality high yield DNA (Qiagen, Hilden, Germany). In short, RBCs were lysed for at least an hour with proteinaseK at 37 °C. Proteins were precipitated out and DNA was extracted using an isopropanol-ethanol extraction. DNA integrity was checked on a 0.8 % agarose gel made with 1x TAE run for 1 hour in 120 V. Ten µg of DNA were digested for at least 16 hours at 37 °C with a combination of three

restriction enzymes (RsaI, HaeIII and HinfI). Samples were then frozen until further processing. When ready for processing, samples were quickly thawed at 37 °C and run on a 0.8 % agarose gel in a pulsed-field gel electrophoresis rig for 19 hours (3 V cm^{-1}, 0.5 s initial switch time and 7 s final switch time) along-side three lanes of 1 kb extension ladder from Invitrogen and two standard lanes made of either Domestic Chicken blood or Tree Swallow blood. The gel was then dried and hybridized overnight with a radioactive probe ('CCCTAA' x 4) that anneals to the single-stranded overhang at the end of the telomere. The next day the gel was washed with a 0.5x SSC solution and placed on a phosphor screen (Amersham Bio-sciences, Buckinghamshire, UK) for at least two days. The screen was then visualized using a Storm 540 Variable Mode Imager (Amersham Biosciences).

Because each cell and each chromosome has a telomere of a different length this procedure results in a smear rather than distinct bands. This smear represents the distribution of telomere lengths (Kimura et al. 2010). Measurements on the telomere distribution were done with ImageJ (Version 2.0.0-rc-34/1.50a; Schindelin et al. 2012), an open source image processing software. Optical density values (OD) were measured along a line centered along each lane. Because one probe molecule attaches to one telomere molecule, the OD values directly correspond to the number of telomere molecules of the length indicated by the position on the gel. The fragment size of each telomere fragment at a given pixel location down the lane (KBi) was measured by fitting a cubic polynomial to the central ladder lane of each gel. We used an analysis window between 1.636 and 40 kb (the two outmost visible size markers). Background was subtracted from all OD measurements and was estimated by measuring a horizontal line placed just below the lowest size marker.

One of the advantages of using the TRF assay over other techniques is that each sample produces a distribution of TL *per individual* rather than one metric that summa-

rizes that distribution (Nussey et al. 2014). This allows us to explore in greater depth how different characteristics of the TL distribution are involved in an individual's physiology. However, statistical methods that can analyze a distribution as one datum, both as a predictor and as a response, are new and still hard to implement (Ramsay et al. 2009). To balance the over-simplification of using only mean TL with the complex statistics involved in using the entire distribution, we measured the following key metrics from each distribution: the mean TL, the skew and kurtosis, and the tenth to ninetieth deciles of the TL distribution. With these metrics we should have captured the main features of each distribution without overly complicating it. However, all these metrics were highly correlated (Table 1.1), and the implications of these correlations for TL measurement will be discussed in a different publication. Because all our metrics were correlated, we reduced the dimensionality of our TL measures with a principle component analysis (PCA) on all 12 metrics. This PCA was conducted using the 'princomp' function from the 'stat' package in R (version 3.2.1). We used only the first principal component score (PC1) for all analyses, as, by itself, it explained 88.5 % of the variation. The loadings for PC1 are presented in the gray row in table 1.1.

Genotyping

To tease apart environmental and genetic effects on TL it is necessary to verify the paternity status of each chick because extra-pair paternity rates in Tree Swallows are high (Conrad et al. 2001). We extracted DNA from RBCs stored in lysis buffer or from dead nestlings. Using polymerase chain reaction (PCR) we amplified nine microsatellite loci that have previously been developed to assess parentage in this species (Makarewich et al. 2009). All nine loci were amplified in two multiplex reactions that have been optimized

Table 1.1: The correlation coefficients (r) between 12 metrics from the TL distribution: mean TL, skew, kurtosis and the 10th to 90th percentiles (P10 to P90 respectively). In all cases $p < 0.001$. The gray row shows the PCA loadings for PC1, which explained 88.5 % of the variation and was thus the only PC used in the analyses for this paper.

	Mean	Skew	Kurtosis	P10	P20	P30	P40	P50	P60	P70	P80	P90
PC1 loadings	0.25	-0.08	-0.49	0.08	0.14	0.18	0.21	0.24	0.27	0.31	0.37	0.49
Mean	1											
Skew	-0.88	1										
Kurtosis	-0.84	0.95	1									
P10	0.72	-0.44	-0.33	1								
P20	0.87	-0.63	-0.52	0.95	1							
P30	0.92	-0.72	-0.61	0.88	0.99	1						
P40	0.95	-0.79	-0.68	0.83	0.96	0.99	1					
P50	0.97	-0.83	-0.74	0.78	0.93	0.98	0.99	1				
P60	0.98	-0.87	-0.79	0.74	0.89	0.95	0.98	0.99	1			
P70	0.99	-0.91	-0.84	0.68	0.85	0.91	0.95	0.97	0.99	1		
P80	0.98	-0.93	-0.9	0.61	0.77	0.84	0.89	0.93	0.96	0.99	1	
P90	0.92	-0.9	-0.94	0.46	0.63	0.71	0.76	0.81	0.86	0.9	0.96	1

for this species. Amplification of PCR products was confirmed by gel electrophoresis prior to fragment analysis using an ABI 3730x1 capillary sequencer. We used the Geneious software (version 9.0.5; Kearse et al. 2012) to assign genotypes for all nestlings and adults, and CERVUS (version 3.0; Kalinowski et al. 2007) to assign parentage. We determined the sex of each nestling by using a P2/P8 sexing protocol with a HaeIII digest similar to that described in Whittingham and Dunn (2000).

Statistical analysis

All analyses were carried out in R (version 3.0.2; R Core Team 2015). We tested linear and generalized linear mixed-effect models using the 'lmer' and 'glmer' functions from the 'lme4' package (version 1.1-11; Bates et al. 2013). We analyzed the determinants of

chick TL by including both parental TL and experimental group in these models. We included in the model the TL of the genetic father and mother but not that of the social or foster parents, so the only extra-pair young that were included in the model were those for which both genetic parents are known (23.5 % of the extra-pair young at this site). To test whether parental and treatment effects vary with the sex of the chick we included an interaction term with sex for each variable. To account for the fact that each parent sired several chicks and that chicks that were born in the same box or grew up in the same box are correlated, we added male and female 'id' and natal and rearing boxes as random intercept effects. The year of study was added as a random effect. Running all models for each year separately reduced our sample size by too much and caused convergence problems for the models. Examining the variance attributed to year in each of the models shows that it is low compared to other random factors and close to zero many times. This suggests year effects, even if they exist, are small.

To test whether parental age predicts chick TL, we added the age of both parents into the full model. For most birds we only have an estimate of their minimum age rather than their known age so the sample size for the known-age model is considerably lower than that for the minimum-age model (31 vs. 120). Using minimum age gives a conservative estimate of the age effect on TL. We ran two separate models, one with the minimum age of the parents as fixed effects (minimum-age model), and one with the known age of the parents (known-age model). The models run with known age were able to give an estimate of the coefficients in the model. However, two circumstances make us doubt these estimates: First, The sample size of 31 was used to estimate 16 parameters. This is obviously a very difficult task, and the models are in danger of being overfit. Second, the model consistently had problems converging on the estimates. While it is hard to say whether these were true convergence problems or false positives it does suggest the sample size was a limiting factor. Despite this, none of the model validation plots showed

any deviation from model assumptions, and the model explained a higher proportion of the variance in chick TL than did the minimum-age model—the marginal R^2 for the known-age model was 0.56 compared to 0.30 for the minimum-age model (based on R^2 calculations developed for mixed models; Nakagawa and Schielzeth 2013). We could not use a likelihood-ratio test to compare models as they were not fit to the same subset of the data. We decided to include both models, and discuss the implications of both the differences between them and the similarities.

These two full models were then simplified by a stepwise-selection process in which we dropped one predictor at a time and compared the AIC values for the full and reduced models. We then sequentially added back previously-dropped terms to verify no effects were erroneously removed from the model. In each step the model that best improved on the previous one (highest ΔAIC) was selected and the process was continued until neither dropping nor adding terms improved the models further.

We estimated the heritability of TL using two different methods: first, we calculated a mid-parent/offspring regression and h^2 was estimated as the slope of this regression. For this analysis we only used cases where we knew both genetic parents. We controlled for the effect of the experimental manipulation by adding it to the model. In addition, we used the minimum-age and known-age models described above to estimate if TL is inherited paternally, maternally or both. Second, we partitioned the total phenotypic variance (V_p) to environmental (V_e) and additive genetic (V_a) components such that $V_p = V_e + V_a$. We estimated variance components by using a REML mixed model with natal and rearing broods as random effects (Kim et al. 2010; Voillemot et al. 2012). As full siblings share 50 % of their genes (Lynch and Walsh 1998), V_a was calculated as twice the variance component attributed to the natal nest. We only used full siblings in this analysis and excluded all extra-pair young. Heritability was estimated as $V_a/(V_a + V_e + V_{res})$,

where V_e is equal to the variance component arising from the nest of rearing and V_{res} is the residual variance component. The effect of the experimental group was controlled for by adding it as a fixed effect in the model. Significance of the random effects was tested using likelihood-ratio tests (LRT) by removing each term and comparing to the full model.

To test the effects of the experimental manipulation on the size of chicks we used a linear mixed-effects model with the interaction of measurement number (out of the four total measurements taken) and experimental group as a fixed effect, and with chick 'id', natal and rearing boxes and year as random effects. P-values for this analysis were obtained using Wald t-tests calculated by the 'lmerTest' package (version 2.0-30; Kuznetsova et al. 2016).

Results

Treatment effect on chick size and mortality

In total, 39 paired-brood manipulations were conducted (16 in 2012, 9 in 2013 and 14 in 2014) and 416 chicks were included in the experiment. At the start of the experiment, when the chicks were between 0 and 2 days old, there was no difference between chicks in enlarged broods and chicks in control broods in mass (control broods: 2.34 ± 0.67 (Mean ± sd); enlarged broods: 2.29 ± 0.60; $t_{101.7} = 0.20$, $p = 0.84$; Fig. 1.1A), wing length (control 6.57 ± 0.78; enlarged 6.49 ± 0.64; $t_{94.6} = 0.14$, $p = 0.89$; Fig. 1.1B) or head-plus-bill length (control broods 13.32 ± 1.03; enlarged 13.23 ± 0.95; $t_{73} = -0.44$, $p = 0.66$; Fig. 1.1C). Chicks

growing up in enlarged broods were smaller than chicks growing up in controls and the difference between them grew with each subsequent measurement (Figs. 1.1A-C). Chicks in enlarged broods had a much smaller probability of fledging (GLMM with binomial family: $\beta \pm sd = -4.89 \pm 1.27$, $\chi^2_1 = 30.58$, $p < 0.001$; Fig. 1.1D). On day 12 male chicks were heavier (Males: 10.00 ± 6.59, Females: 9.26 ± 6.09; $F_{187.01} = 6.90$, $p = 0.009$) and had a longer head-plus-bill (Males: 19.39 ± 4.55, Females: 18.88 ± 4.43; $F_{186.01} = 6.08$, $p = 0.01$). Males also had slightly longer wings but this did not reach statistical significance (Males: 21.31 ± 15.41, Females: 19.97 ± 14.34; $F_{171.91} = 1.30$, $p = 0.26$).

The effect of the brood enlargement

The brood enlargement came out as an important predictor of chick TL only in the minimum-age model (see below). Under the minimum-age model chicks in enlarged broods had shorter TL than did those in control broods but, while this effect stayed in the final model based on AIC, it did not reach statistical significance based on the F-test (Chicks in control broods 1.04 ± 3.91 (mean ± sd); enlarged broods 0.30 ± 3.94; $F_{25.68} = 2.54$, $p = 0.12$; Fig. 1.2A).

It is possible that the small treatment effect we observe is due to selective mortality of short-telomere chicks, which died before their TL could be sampled. Although we do not have TL samples from chicks who died before day 12, if we look at the probability of fledging in all chicks for which we have a telomere sample, we see that short-telomere chicks are not less likely to fledge than are long-telomere chicks ($\beta = -0.05 \pm 0.06$, $\chi^2_1 = 0.65$, $p = 0.42$).

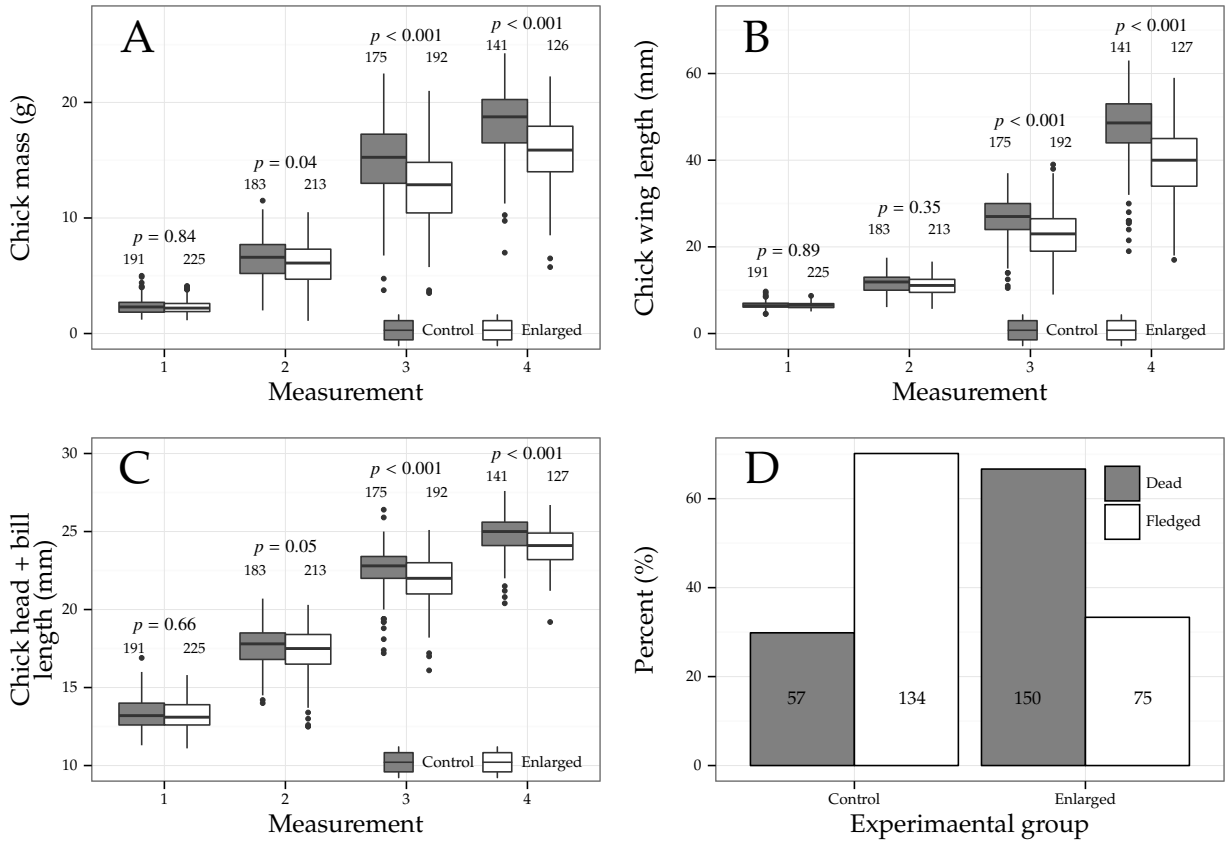

Figure 1.1: The effect of the brood enlargement on chick mass (A), wing length (B), head-plus-bill length (C) and probability of fledging (D). Panels A-C show boxplots of the change in size with each measurement (out of four taken) in control (dark boxes) and enlarged (light boxes) broods. Dots are outliers. The numbers above each box are the sample size and the p-value for the effect within each measurement is shown above the sample sizes. Panel D shows the percent of chicks that fledged (light bars) or died (dark bars) in either control (left side) or enlarged (right side) broods. The numbers in each bar are the sample sizes. All chicks, including ones that died before the age of 12 days, are shown.

Minimum-age model

After the stepwise-selection process the best minimum-age model included sex, experimental group, the TL of the genetic father and the interaction of chick sex and the TL of the genetic mother. Chick sex was included in the best model as a main effect because it interacted significantly with mother TL but had no net effect on TL (Females 0.80 ± 4.01

(mean ± sd); males 0.90 ± 3.77; $F_{107.85} = 0.01$, $p = 0.90$; Fig. 1.2B).

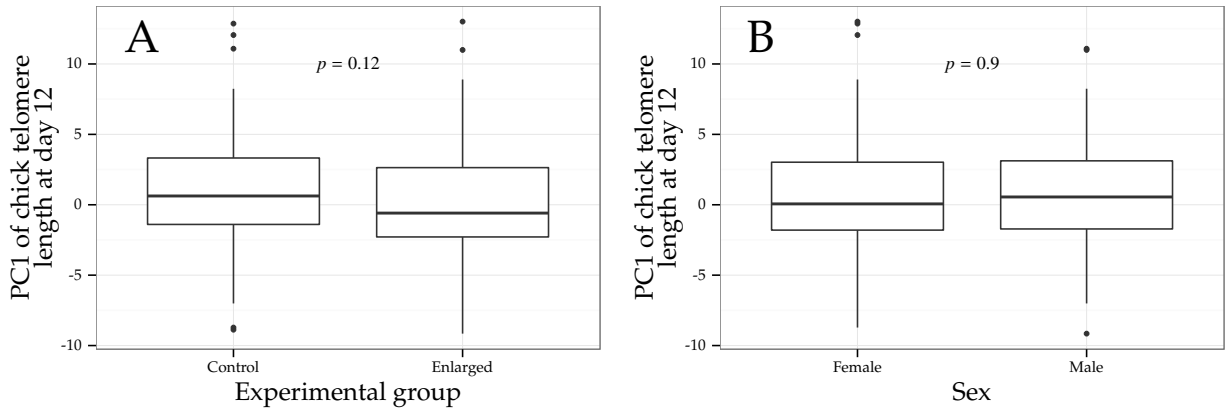

Figure 1.2: The effect of the brood enlargement (A) and chick sex (B) on chick telomere length. Dots are outliers. The number above the boxes is the p-value based on an F-test.

The TLs of both the mother and father were important predictors of chick TL (Fig. 1.3). Mother TL interacted with chick sex such that the correlation between a mother's TL and her daughter's TL was higher than that with her son's (Mother-Daughter: $\beta = 0.68 \pm 0.16$; Mother-Son: $\beta = 0.40 \pm 0.16$; $F_{62.02} = 9.11$, $p < 0.001$; Fig. 1.3A). Father TL was equally correlated with that of his sons and daughters (Father-offspring: $\beta = 0.23 \pm 0.13$; $F_{34.55} = 3.00$, $p = 0.09$; Fig. 1.3B).

Known-age model

When the known age of each adults was substituted for minimum age (known-age model) the best model included father TL ($\beta = 0.69 \pm 0.25$, $F_{30.58} = 7.23$, $p = 0.01$;Fig. 1.4), father age ($\beta = -2.08 \pm 0.58$, $F_{25.38} = 12.73$, $p = 0.001$; Fig. 1.5A) and mother age ($\beta = 1.21 \pm 0.37$, $F_{11.15} = 10.51$, $p = 0.008$; Fig. 1.5B).

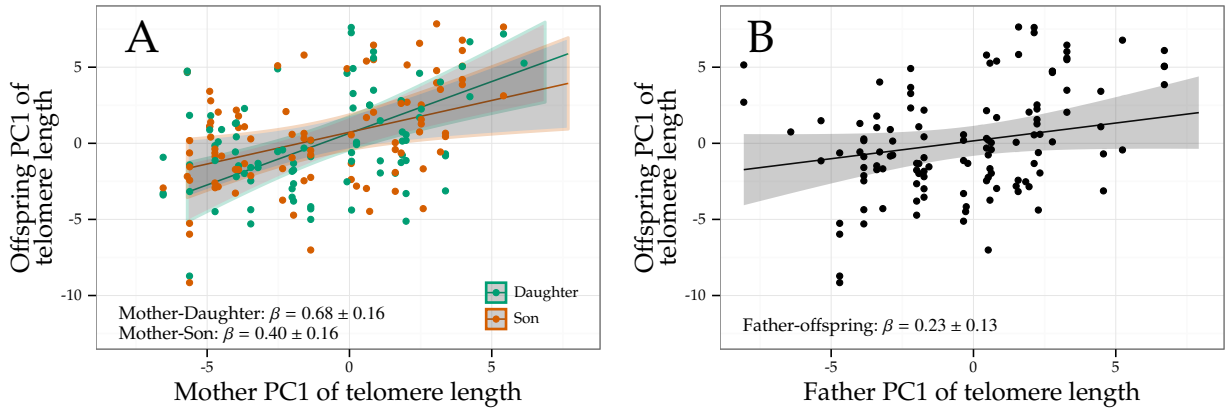

Figure 1.3: Telomere length (TL) heritability patterns for both mothers (A) and fathers (B). Panel A shows that mother TL is more correlated with that of her daughters (green) than that of her sons (orange). The interaction of mother TL and chick sex is highly significant ($F_{62.02} = 9.11, p < 0.001$). Father TL does not show a significant difference in heritability between sons and daughters so only the general regression line is shown (B). While father TL came out as an important predictor of chick TL based on AIC model selection it is only borderline significant based on the F-test ($F_{34.55} = 3.00, p = 0.09$)

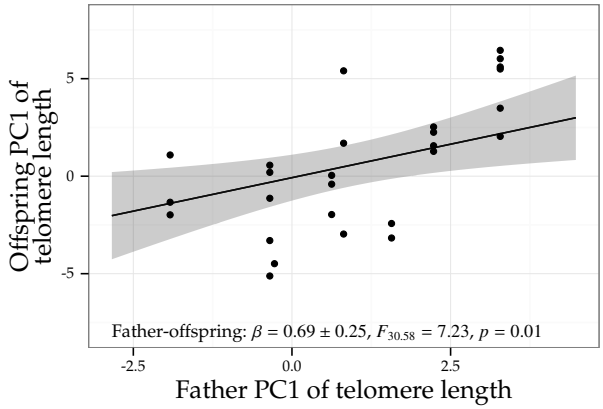

Figure 1.4: The effect of father telomere length on chick telomere length under the known-age model.

Heritability estimates

The average TL of the genetic parents was highly correlated with the TL of all within-pair offspring of those parents and the h^2 was estimated as 0.81 (LMM: $\beta = 0.81 \pm 0.17$, $F_{42.48} = 22.47$, $p < 0.001$; Fig. 1.6). The 95 % confidence interval for this estimate is 0.43

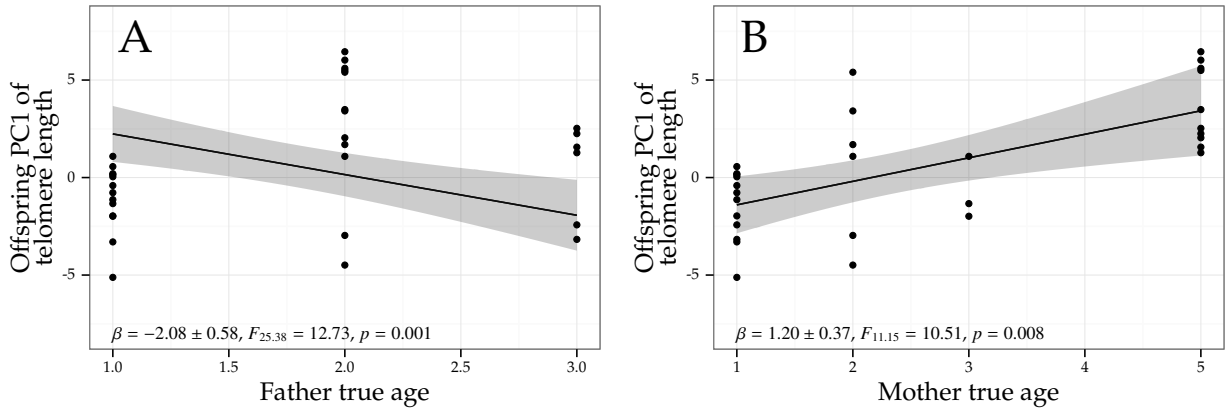

Figure 1.5: The effect of father (A) and mother (B) known age on chick telomere length.

to 1.17. To not lose information by averaging TL values per brood, all chicks were used in this analysis and the natal and rearing boxes were added as random effects. However, if we average all the within-pair offspring and repeat the analysis, we still get a high estimate of h^2 ($\beta = 0.78 \pm 0.19$, $F_{35} = 16.18$, $p < 0.001$)

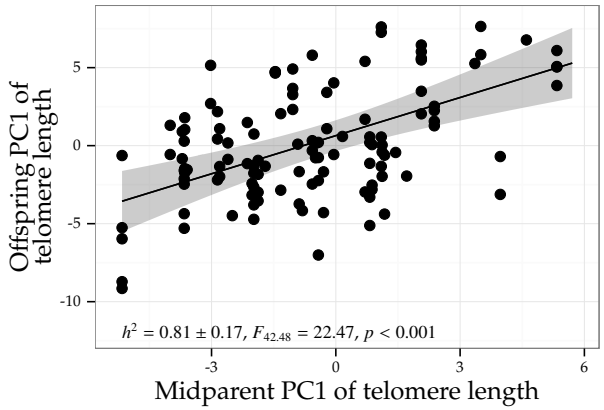

Figure 1.6: The regression of chick telomere length on mid-parent telomere length. The slope of this regression, the h^2 value is 0.81. Experimental group was added as a fixed effect to control for any effect of the manipulation.

Analysis of the variance components attributed to natal and rearing nest showed significant additive genetic variance (i.e., attributed to nest of origin, variance = 11.29 ± 0.32, $\chi^2_1 = 35.65$, $p < 0.001$) and environmental variation (attributed to nest of rearing, variance = 1.59 ± 0.12, $\chi^2_1 = 3.00$, $p = 0.08$). These variance components can then be

translated to a h^2 estimate (Kim et al. 2010; Voillemot et al. 2012). The model estimates V_a as 22.58 (twice the variance attributed to nest of origin) and V_e as 1.59 (the variance attributed to nest of rearing). Together with the residual variance estimate of 2.28 this translates to a h^2 estimate of 1.25.

Discussion

In this study we evaluated the relative roles of inheritance and early environment in affecting Tree Swallow telomere length, to better understand the determinants of early-life TL. Our results indicated a strong role for inheritance and a weak effect of brood enlargement on chick TL. In addition, we did not find a sex effect on TL in 12-day-old chicks. In most TL studies conducted on chicks, no difference between the sexes has been found (Caprioli et al. 2013; Nettle et al. 2015; Parolini et al. 2015; Young et al. 2013; but see Foote et al. 2011b). Parallel results from the same Tree Swallow population have shown that adult males have longer telomeres than females and that this difference develops before the first breeding attempt (Belmaker 2016, chapter 3), suggesting that the sex differences in TL develops during sexual maturation, before the commencement of breeding, and are maintained thereafter. A few hypotheses have been put forward to explain the sex-difference in TL (reviewed in Barrett and Richardson 2011). Heterogamy, genetic imprinting, sex-differences in growth rate and different hormonal balance have all been suggested to contribute to the development of the sex-difference in TL (reviewed in Barrett and Richardson 2011). However, to date, no one hypothesis can explain the full variation of sex effects on TL observed in nature (Barrett and Richardson 2011). This is an important avenue to pursue but more research is needed before we can find general patterns and causes .

Brood enlargement

Our results show a weak effect of the brood enlargement on TL of 12-day old chicks. The direction of the manipulation effect is as predicted (shorter telomeres in enlarged broods) and experimental group did come out as an important predictor of chick TL in the minimum-age model. However, the F-test for experimental group in this final model did not quite reach statistical significance and the observed effect size is small (Fig. 1.2A). Telomeres shorten as a consequence of increased stress (Epel et al. 2004), and most telomere shortening happens early in life (Haussmann and Marchetto 2010). It has been shown that catch-up growth, as experienced by small chicks in large broods, can result in faster telomere attrition (Geiger et al. 2012; Reichert et al. 2015b). Given the evidence that early environment is an important factor in creating variation in TL (Nettle et al. 2015), it is surprising that our experimental manipulation had such a small effect on TL.

In this study chicks were sampled for TL at the age of 12 days. Chicks that died before reaching that age were not sampled, as the TRF assay is sensitive to DNA degradation and one cannot use DNA from dead chicks (Haussmann and Mauck 2008a). One possibility is that short-telomere chicks did not survive to be sampled and were overrepresented among these dead chicks, causing the lack of effect on TL we observed. According to this scenario only long-telomere chicks, which are higher quality (Heidinger et al. 2012), survive to sampling age. Those high-quality chicks would be less affected by the stress of the manipulation. As we do not have telomere samples from chicks that died before sampling, we cannot rule out this possibility. However, when testing the survival effect of TL from day 12 onward we see there is no effect of TL on the probability of fledging. Even though this result does not conclusively show that the results are not biased by selective mortality of short-telomere chicks, it does suggest that there are other factors at work here.

Chicks in enlarged broods were substantially smaller than those in control broods, and they had a smaller probability of fledging (Fig. 1.1). Thus, the treatment was effective in creating a competitive environment for the chicks. However, this setting did not induce much variation in TL. It is possible that the measured effect of the brood enlargements on TL was weak because we measured the chicks at the age of 12 days while Tree Swallow chicks fledge closer to the age of 21 days–the chicks still had a long time to experience the manipulation and suffer any effects of increased competition on TL. In addition, the period immediately post-fledging is a major source of stress for fledglings, especially in swallows as they learn how to forage independently (Naef-Daenzer et al. 2011). Even though the chicks would have completed most of their growth by 12 days of age (Winkler and Adler 1996) it is possible that, had we measured the chicks closer to fledging, or even post-fledging, we would have seen a larger difference between the experimental groups. The choice of sampling age was made to minimize the chances of premature fledging, and this is a constraint of work with box-nesting swallows. In any event, 12 days of elevated competition during the most active phase of chick growth is apparently not enough to induce noticeable telomere shortening.

Voillemot et al. (2012) conducted a similar brood enlargement on wild Collard Flycatchers (*Ficedula albicollis*) and also did not find any effect of the manipulation on the TL of the chicks at 12 days of age. Reichert et al. (2015b) enlarged broods of captive Zebra Finches (*Taeniopygia guttata*) and, while after 10 days they did not see any differences in TL between groups, by day 45, chicks in enlarged broods had shorter telomeres. However, 12 days in a competitive environment *were* enough for European Starlings (*Sturnus vulgaris*) chicks to develop shorter telomeres (Nettle et al. 2015). Taken together, this suggests that harsh conditions need to be sustained in time to have an effect on TL. If the harsh conditions experienced by a brood are restricted in time, the chicks may be able to avoid long-term TL shortening. We did see a trend in the direction of stress-induced

telomere shortening, but one that is not quite significant. This small difference may then grow larger as the chicks continue to mature toward fledging and post-fledging.

Sex, age and heritability estimates

Two main differences were found between the minimum-age model and the known-age model. First, in the minimum-age model parent TL was found to be an important predictor of chick TL, with a stronger maternal inheritance (Fig. 1.3). We cannot exclude the possibility that maternal effects before hatching, such as egg composition or incubation patterns, are a cause of the high sibling correlation. Egg swapping should be used to further investigate that option, but will not control for maternal effects during egg maturation. In contrast, in the known-age model only paternal inheritance was found (Fig. 1.4). Second, no effects of parent age were found in the minimum-age model while age was found to be important in the known-age model (Fig. 1.5).

It is possible that the parental age effect and the parental TL effect are related and some how mask each other. Before development starts, the zygote inherits its telomeres from the gametes of the parents (De Meyer et al. 2014; Graakjaer et al. 2004). The TL of the *specific* sperm and egg forming the zygote will determine its TL—a parental TL effect on chick TL. Any age-specific process to affect sperm and egg TL will determine the pool from which gamete telomeres can be chosen, and will consequentially affect offspring TL—an age effect on chick TL. This suggests the parental TL and parental age effects are related and, if indeed they mask each other, it would explain why parental TL appears as important in the minimum-age model while parental age appears as important in the known-age model. While there might not be anything specific about minimum and

known-age that will favor the appearance of one versus the other, sample sizes and noise could determine which effect comes out as important in any given model.

A few non-mutually-exclusive processes determine how the distribution of TLs in the gametes changes with age. First, within an individual's life, TL is known to shorten with age (Hall et al. 2004; Haussmann et al. 2003; Haussmann and Marchetto 2010). However, telomerase, the enzyme that lengthens telomeres, is active in the germline of adult birds (Haussmann et al. 2004; Haussmann et al. 2007), and might act to counter this age-related shortening. Second, while sperm quality has been shown to diminish with age (Van Waeleghem et al. 1996) and a low sperm count and low sperm quality are associated with short TL (Ferlin et al. 2013; Rocca et al. 2016), sperm TL has been shown at least once to increase with age, possibly due to selective stem cell turnover (Kimura et al. 2008). Lastly, stochastic processes during sperm maturation will increase variability in sperm TL as the individual ages (De Meyer and Eisenberg 2015). These processes are only relevant in males and not in females, as sperm are produced continuously while eggs are generally produced only once (De Meyer and Eisenberg 2015). On a population level, TL has been shown to be a good proxy for individual quality (Bauch et al. 2013; Le Vaillant et al. 2015). Selective mortality of short-telomered individuals will increase mean TL in older individuals (Haussmann and Mauck 2008b). This last mechanism is relevant to both males and females. These processes do not operate independently, and the sum of their effects will determine the distribution from which the TL of each gamete is chosen. Because the relative importance of each will vary with the specific conditions of each study, many possible patterns of parental-age effects are possible. Here we find that male age is *negatively* associated with chick TL, while female age is *positively* related to it. In humans, the general pattern is that of male age positively correlating with TL of the children (Arbeev et al. 2011; Broer et al. 2013; De Meyer et al. 2007; Eisenberg et al. 2012; Ferlin et al. 2013; Kimura et al. 2008; Nawrot et al. 2004; Prescott et al. 2012; Unryn et al. 2005) but a

positive effect of mother age can also be found (Ferlin et al. 2013). In Sand Lizards (*Lacerta agilis*) paternal age is negatively associated with offspring TL (Olsson et al. 2011). In Great Reed-Warblers (*Acrocephalus arundinaceus*) heritability is maternal, as is the positive age effect (Asghar et al. 2015b).

That these processes occur mostly in males would predict some form of sex-specific heritability pattern for TL (De Meyer and Eisenberg 2015), as the pool from which sperm and egg are chosen varies with age in a sex-specific way (De Meyer and Eisenberg 2015). Indeed many studies show that TL heritability is sex-specific (i.e., Njajou et al. 2007), but the exact pattern, whether female of male biased, seems to be study-specific (Broer et al. 2013). It has been suggested that a combination of genetic imprinting and heterogamy could cause this sex-specific pattern (Reichert et al. 2015a), but the evidence in favor of this hypothesis is mixed (Broer et al. 2013; Eisenberg 2014). Our results show that inheritance is maternal and that the TL of the mother is more strongly related to the TL of her daughters than of her sons, suggesting linkage to the W chromosome. However, in Sand Lizards (*Lacerta agilis*), where females are also the heterogametic sex, father-son correlations are stronger than mother-daughter correlations, suggesting a Z-linkage and paternal inheritance (Olsson et al. 2011). In humans, both maternal as well as paternal TL inheritance is often found (Broer et al. 2013; Eisenberg 2014), and there is evidence for X-linked TL heritability (Nawrot et al. 2004). TL heritability patterns might be study-specific as a consequence of biological or methodological factors (De Meyer and Eisenberg 2015), and it has been suggested that some of the observed variation in the sex-specificity pattern of TL heritability is not biological in origin (Eisenberg 2014).

The fact that most studies that look at the heritability of TL find some form of sex-specificity is intriguing and deserves attention. However, no general pattern has been found yet to explain variation in sperm TL and the sex-specific inheritance pattern of TL

(Broer et al. 2013). It is not clear whether one such cause exists or several causes interact to create the variation in heritability patterns we see (De Meyer and Eisenberg 2015). What is clear it that more studies are needed on telomere dynamics in the germline, throughout an individual's life, in both sexes, to better understand how TL is inherited and how an individual's age and sex affect TL heritability patterns.

Modes of TL inheritance

Two mechanisms contribute to the correlation in TL between parents and offspring: on the one hand TL is inherited directly from the gametes of the parents (De Meyer et al. 2014; Graakjaer et al. 2004), and throughout the subsequent life of the zygote as it develops into a hatchling then an adult, decreases in its TL can be countered by TL maintenance mechanisms that are also inherited from the parents (Hjelmborg et al. 2015). Heritable variation in both the base telomere sequence and the telomere repair mechanisms can help produce correlations between parents and offspring. If we were to measure TL in chicks soon after hatching, the influence of the base telomere sequence inherited from the parents would dominate any inherited similarity based on shared telomere-maintanence genes (De Meyer et al. 2014). In contrast, if chicks are measured when they are older, the environment will have had a chance to decrease the chick's TL and in-born repair mechanisms can act on any such erosion. Thus, as the chicks get older, the stochastic nature of environmental challenges to their TL, together with genetic variation in the effectiveness of repair mechanisms, can present many avenues to reduce the similarity between parents and offspring. A recent study in King Penguins (*Aptenodytes patagonicus*) shows this exact pattern: TL was found to be maternally inherited when the chicks were 10 days old but there was no significant heritability at older chick ages (Reichert et al.

2015a).

The important point here is that TL is a dynamic character. When we compare the TL of parents and offspring, we are comparing measures at two very different life stages where the relative importance of environmental stressors and inherited influences are going to be different. Because both the initial telomere sequence and mechanisms of telomere repair are inherited, a correlation between parents and offspring might be expected at any combination of their relative ages, but that underlying similarity is going to be arising through very different pathways as the trait is measured in individuals of different relative age.

It is important to bear this in mind when considering natural selection's ability to shape TL variation. Telomere shortening *rate* has been shown to affect fitness irrespective of telomere *length* (Bize et al. 2009; Epel et al. 2009; Salomons et al. 2009), and shortening rate has been shown to be heritable as well (Hjelmborg et al. 2015). Inheritance of a long-telomere base sequence can give an individual an early advantage, but without a good mechanism to maintain those long telomeres, an individual will suffer the deleterious effects of telomere erosion. An individual born with short telomeres but with an efficient telomere-maintenance system can still benefit greatly from keeping its telomeres from shortening further. When we try to estimate natural selection's ability to mold TL variation, we need to keep in mind that the inheritance of telomerase activity and genes responsible for dealing with stress and other telomere-maintenance genes may be at least as important as initial TL in affecting fitness differences among individuals.

Conclusion

TL in Tree Swallows shows high sex-specific heritability and a weak effect of early-growth environment. While it seems that natal competition must be very strong or chronic to shorten telomeres considerably, we believe that the negligible effect sizes detected are a consequence of the relatively early developmental period in which we measured TL and that stress will likely be more important as a cause of TL variation as chicks mature.

CHAPTER 2

THE EFFECTS OF BROOD-ENLARGEMENT ON THE TELOMERE LENGTHS OF WITHIN- AND EXTRA-PAIR CHICKS IN TREE SWALLOWS (*Tachycineta bicolor*)

Abstract

The "good-genes" hypothesis to explain female extra-pair (EP) mating states that females benefit from this behavior by having higher-quality offspring. However, despite extensive research, the predictions of the good-genes hypothesis have been confirmed in only half of published papers. One possibility to explain this discrepancy is that the benefit of extra-pair copulation (EPC) is context-dependent. To test this hypothesis we use telomeres, the protective caps of chromosomes, and look at the telomere length (TL) of extra and within-pair offspring (EPO and WPO respectively) growing up in either control or enlarged broods. If EPO are indeed better than WPO, and this difference is context-dependent, we predicted that the TL of EPO will be longer than that of WPO but only in enlarged broods. EP status did not predict TL or size either alone or in isolation. As predicted, EPO had a higher probability of fledging than WPO but only in enlarged broods, but this effect was only seen after a separate analysis per group. These results provide some support for the good-genes hypothesis. The lack of effect of EP status on TL could be either because TL was sampled too soon in development, because TL and brood enlargement are not the appropriate metric and context for this test, or because we lack the ability to detect such a benefit.

Introduction

Before the revolution in biology caused by the development of modern molecular techniques, most passerine species were considered both socially *and* genetically monogamous (Lack 1968). However, after it became possible to easily and cheaply genotype many individuals in the wild (Jeffreys et al. 1985), it became clear that extra-pair copulation and paternity (EPC and EPP respectively), in which a female mates with a partner other than her social mate, is common (Griffith et al. 2002). Attempts to answer why females mate with more than one male have yielded a myriad of hypotheses (reviewed in Akçay and Roughgarden 2007; Griffith et al. 2002). While it has been suggested that female EPC behavior is not adaptive (Forstmeier et al. 2014), there are many examples where a benefit to female EPC has been found (Akçay and Roughgarden 2007). In addition, the fact that EPC is costly to the female (Ferretti and Winkler 2009) suggests that there is a benefit of some kind that keeps this behavior in the population. In those cases where we cannot show that EPC yields a benefit, either direct or indirect, it is worthwhile to ask if the behavior is really not adaptive or whether we just lack the ability to detect its benefit.

Females have been hypothesized to engage in EPC behavior to guard against infertility in their social mate (Sheldon 1994; Wetton and Parkin 1991), to increase the genetic diversity of her brood (Westneat et al. 1990; Williams 1975), or to induce the EP sire to help in rearing the offspring (Burke et al. 1989; Colwell and Oring 1989; Townsend et al. 2010; Wolf 1975). But the hypothesis that has received the most attention states that females that participate in extra-pair matings gain "good genes" for their offspring, either directly, by mating with a male superior to their social mate (Birkhead and Moller 1992; Hamilton 1990; Møller 1988; Westneat et al. 1990), or by choosing an extra-pair sire with

complementary genes (Kempenaers et al. 1999; Tregenza and Wedell 2000). The 'good genes' hypothesis predicts that extra-pair sires will be of higher quality than social mates and/or that extra-pair offspring (EPO) will be of better quality than within-pair offspring (WPO) (Griffith et al. 2002). However, despite years of extensive research to evaluate this hypothesis, results are mixed at best (Hsu et al. 2014; Hsu et al. 2015), and the predictions of the good-genes hypothesis have been born out in only 50 % of published papers (Akçay and Roughgarden 2007).

One potential explanation for the fact that good-genes predictions have been met only half of the time is that the benefits of EPC are evident only in certain environments and not others (Schmoll 2011). For example, EPO may inherit genes that give them a competitive advantage in the nest, allowing them to grow even when food is scarce. However, when conditions are good, such as in a brood of modal size or smaller when food supplies are rich, this advantage will not manifest itself, as there is reduced competition for food in such an environment. EPO in this scenario will appear to be of equal quality to WPO. In contrast, larger-than-normal broods will force the chicks to compete for parental resources. In this case EPO can take full advantage of their better genes to out-compete their WPO half siblings, and EPO will be seen to be better than WPO. While this is just a hypothetical example, it illustrates well how the environment can interact with the genetic makeup of offspring, and how this interaction can obscure the benefits of EPC to the female.

Despite its potential for explaining why benefits of EPC to the female are found only in some studies and not others, this context-dependence hypothesis has been directly tested only a few times. In Coal Tits, *Periparus ater*, EPO had a higher probability of recruiting into the population than WPO, but only late in the season (Schmoll et al. 2005). The humoral immune response of Blue Tit chicks, *Cyanistes caeruleus*, was stronger for

EPO, but only in artificially enlarged broods (Arct et al. 2013). Lastly, year-effects, where differences between EPO and WPO are seen only is some years, were found for both Yellowthroat, (*Geothlypis trichas;* Garvin et al. 2006), and House Wrens (*Troglodytes aedon;* Forsman et al. 2008).

These studies provide evidence for the environmental dependency of the good-genes hypothesis and suggest that challenging or stressful environments bring out differences between WPO and EPO (but see O'Brien and Dawson 2007). These studies demonstrate a context-dependent advantage to EPO in one or a few traits; however, they do not show an effect on fitness as a whole; a competitive advantage in growth might not equate to a competitive advantage in other important aspects such as predator evasion, for example. Thus, EPO, who show an advantage in one trait, might not be of higher quality at all when we look at lifetime reproduction and survival. It is not readily apparent how to choose the appropriate fitness proxy and/or context to test for good-genes effects. What is needed is a proxy of fitness that integrates a large proportion of an individual's physiology and is predictive of fitness. Telomere length (TL) might prove to be useful in this respect (Monaghan and Haussmann 2006).

Telomeres are the protective caps of eukaryotic chromosomes (Harley 1991). They shorten with each cell replication, as a consequence of the inability of the DNA polymerase to replicate the tips of chromosomes (Levy et al. 1992). Telomeres can be shortened further by oxidative stress (Epel et al. 2004) and the activation of the vertebrate stress response (Saretzki and Von Zglinicki 2002). For this reason telomeres can be seen as an integrator of physiological and environmental stress—stressed individuals have shorter telomeres (Haussmann and Marchetto 2010). When telomeres get too short they cannot preform their protective function any more, which starts a physiological chain-reaction that can lead in the extreme to reduced function and mortality of the individual (Campisi

2005). For this reason short TL is associated with low survival in many species (reviewed in Haussmann and Marchetto 2010), is related to many pathologies (Blasco 2005) and is commonly used as a proxy for quality (Bateson 2016; Bauch et al. 2013; Le Vaillant et al. 2015). In addition, early-life TL has been shown to be a better predictor of survival than late-life TL (Heidinger et al. 2012; but see Caprioli et al. 2013) so any effects of extra-pair (EP) status on TL in chicks can be inferred to be connected to longer term fitness effects. While the exact causal links between TL, survival and performance are not yet clear (Simons 2015) we can still use TL as a *marker* for fitness and performance. Because many physiological processes affect TL it can be used as an integrative measure of a key component of fitness, namely survival, and it allows us to generalize over many physiological aspects of fitness rather than focus on only a few.

To test if the benefit to female EPC behavior is context-dependent we manipulated a wild population of Tree Swallows, *Tachycineta bicolor*. In Tree Swallows, short TL is associated with low survival (Haussmann et al. 2005) and shortens with age (Haussmann et al. 2003; but see Belmaker 2016, chapter 3). In addition, telomerase, the enzyme that is responsible for lengthening telomere, is activated in Tree Swallow chicks but is down-regulated in adults (Haussmann et al. 2004; Haussmann et al. 2007). Not much else is known about telomere dynamics in this species (Belmaker 2016). In contrast, much is known about EPP in Tree Swallows. EPP rates in Tree Swallows are very high (Barber et al. 1996; Conrad et al. 2001), and females are in control of fertilizations (Lifjeld and Robertson 1992; Lombardo 1986; Venier et al. 1993). This suggests that female EPC in Tree Swallows is adaptive. However, the nature of the benefit females get from EPC is still unclear (Whittingham and Dunn 2001). A couple of studies found suggestive variation in semen characteristics, providing weak evidence for the fertility-insurance hypothesis (Lombardo et al. 2002; Lombardo et al. 2004). Some studies have shown that females choose older (Bitton et al. 2007) and higher quality EP sires (Bitton et al. 2007; Kempe-

naers et al. 2001; Whittingham and Dunn 2014; Whittingham and Dunn 2016), in support of the good-genes hypothesis. However, other studies do not find such support for good-genes (Barber et al. 2005; Dunn et al. 2009; Kempenaers et al. 1999; Stapleton et al. 2007; Whittingham and Dunn 2010; Whittingham et al. 2006). We can see that, mirroring the trend in the literature, the good-genes hypothesis in Tree Swallows is only supported in some studies and not others. Together with the population differences in the traits characterizing EP sires (Whittingham and Dunn 2014), this suggests that the advantage to EPC might be context-dependent (Schmoll 2011).

Here we take advantage of a brood enlargement aimed at studying the determinants of early-life TL (Belmaker 2016, chapter 1) to compare the TL of EPO and WPO growing up in control vs. enlarged broods. If the predictions of the good-genes hypothesis are true, and EPO are indeed better than WPO, we would expect EPO to have longer TL than WPO, as early-life TL is a predictor of survival (Heidinger et al. 2012). If the benefit to EPO is context-dependent (Schmoll 2011) we predict that the TL of EPO will be longer than that of WPO but only in enlarged broods.

Methods

Experimental procedure

To evaluate the effects of EP status and context on chick TL we used a brood manipulation that also evaluated the determinants of early-life TL. For further details of the study system, experimental manipulation and the measurement of TL see Belmaker (2016, chapter

1).

For three breeding seasons from 2012 to 2014 we created control and enlarged broods of Tree Swallows, *Tachycineta bicolor*, and cross-fostered chicks between them. Tree Swallows are small migratory aerial insectivores that have been used extensively as a model system for studies ranging from physiology to life history (Jones 2003). In each year we matched broods for hatch date, female age and clutch size. We then randomly swapped about 50 % of the chicks between the two broods and added chicks to the enlarged brood of the pair. Chicks for the brood enlargement came from a nest not participating in the experiment. We then followed the nesting attempt to completion, measuring the chicks every four days for mass, wing length and head-plus-bill length. On day 12 we took a blood sample for both TL measurement and genotyping. Adults were caught during incubation or chick rearing and a blood sample for genotyping was taken. After day 12 the nest was not disturbed until all chicks fledged. Once the last chick fledged, any dead chick that remained in the box was noted and collected for genotyping.

TL analysis was conducted using the TRF assay, which produces a distribution of TLs per sample (Kimura et al. 2010). For each TL distribution we measured the mean TL, skew, kurtosis and the 10th to 90th deciles. These 12 metrics were combined using a principle component analysis (PCA) and the first principal component (PC1) was used as our measure of TL. The details and rationale for this approach are described in Belmaker (2016, chapter 1). Genotyping was done using PCR amplification of nine microsatellite loci that have previously been developed to assess parentage in this species (Makarewich et al. 2009). We used Geneious software (version 9.0.5; Kearse et al. 2012) to assign genotypes for all nestlings and adults, and CERVUS (version 3.0; Kalinowski et al. 2007) to assign parentage.

Statistical analysis

All analyses were carried out in R (version 3.0.2; R Core Team 2015). We analyzed linear and generalized linear mixed-effect models using the 'lmer' and 'glmer' functions from the 'lme4' package (version 1.1-11; Bates et al. 2013). We built models for five response variables: TL, mass, wing length, head-plus-bill length and probability of fledging. In each model we added EP status, experimental group and their interaction as fixed effects. To control for the cross-fostering procedure, both natal and rearing broods were added as random intercepts in our model. In addition, year and the identity of both genetic parents were also added as random effects. Year was not added as a fixed effect because the sample size per year was low enough that it caused convergence problems in the models when included as a fixed effect.

Each chick was measured a maximum of four times (ages zero to 12) but as not all chicks in a brood hatched the same day, their true age is uncertain. For this reason we used the number of each measurement (one to four) as our metric for age rather than their true age in days. For all three size measures (mass, wing length and head-plus-bill length) we added the effect of chick age by adding the measurement number as a fixed effect. This resulted in a 3-way interaction of status, experimental group and measurement. In these three cases chick id was added as a random factor to account for the repeated measures per chick. In essence, we expanded on the model used by Belmaker (2016, chapter 1) and added status as a predictor of growth. Only one sample was taken for TL (on day 12), so the TL model included only samples from chicks that survived to that age. The model for the probability of fledging included chicks for which we do not have a telomere sample.

The models were then simplified using a backward selection procedure, using

AIC values as a criterion for exclusion. In each step we dropped each term sequentially and compared the AIC values of the full and reduced models. The term with the highest ΔAIC was dropped from the model. The process was then repeated until no further model simplification could improve the AIC. P-values for the final model were obtained using likelihood-ratio tests.

Results

In total, 39 paired-brood manipulations were conducted (16 in 2012, 9 in 2013 and 14 in 2014), and 416 chicks were used in the experiment. Within-pair young were slightly lighter at the start of the experiment (mass of EPO: 2.27 ± 0.60 (mean ± sd), WPO: 2.16 ± 0.55; $\beta = -0.19 \pm 0.08$, $\chi^2_1 = 4.68$, $p = 0.03$), and had slightly shorter wings (wing length of EPO: 6.47 ± 0.66 (mean ± sd), WPO: 6.37 ± 0.67; $\beta = -0.19 \pm 0.11$, $\chi^2_1 = 2.48$, $p = 0.11$). The latter effect was evident using AIC but was not significant when using the likelihood ratio tests. The interaction of EP status and experimental group was not found to be an important predictors of TL (Fig. 2.1), mass, wing or head-plus-bill.

The best model for the probability of fledging included the predictors experimental group and EP status but not their interaction ($\chi^2_1 = 0.72$, $p = 0.39$). However, the ΔAIC between the full model with the interaction and the reduced model without it was only 1.28 meaning both models are equally informative. In addition, figure 2.2 shows a possible interaction between EP status and experimental group. We thus ran a separate analysis of the probability of fledging by experimental group, which shows that the independent effect of EP status and experimental group on the probability of survival is driven entirely by WPO in the enlarged broods (control broods: $\chi^2_1 = 2.30$, $p = 0.13$;

enlarged broods: $\chi^2_1 = 8.75$, $p = 0.003$; Fig 2.2).

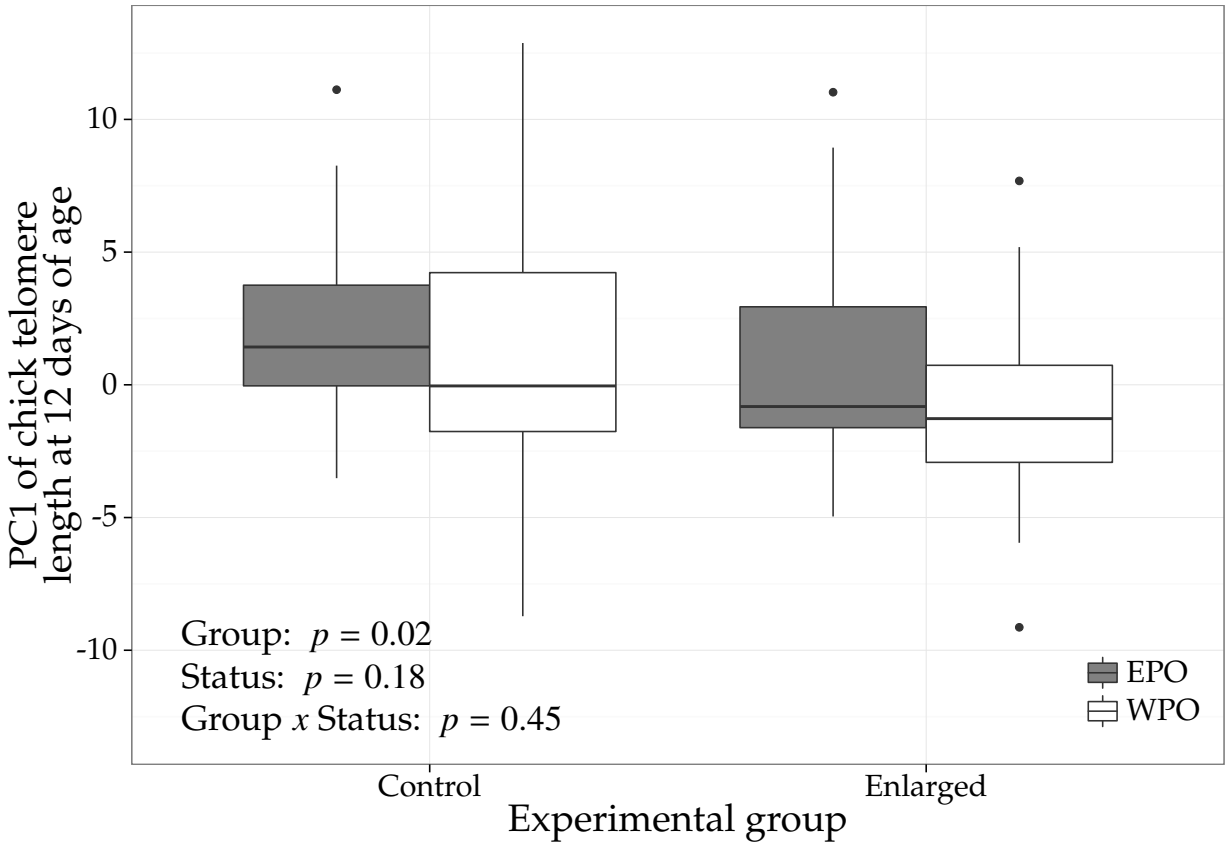

Figure 2.1: The TL of 12-day-old within-pair (WPO, light boxes) and extra-pair (EPO, dark boxes) chicks growing up in control (left side) or enlarged (right side) broods. Dots represent outliers.

Discussion

In this study we tested the variant of the good-genes hypothesis that predicts that EPO are higher quality than WPO, but that this difference is context-dependent and only manifested in stressful environments (Schmoll 2011). We used TL as an indicator of the quality of chicks and tested whether EP chicks had longer TL than did WP chicks in enlarged broods and not in control ones. The experimental treatment succeeded in creating a

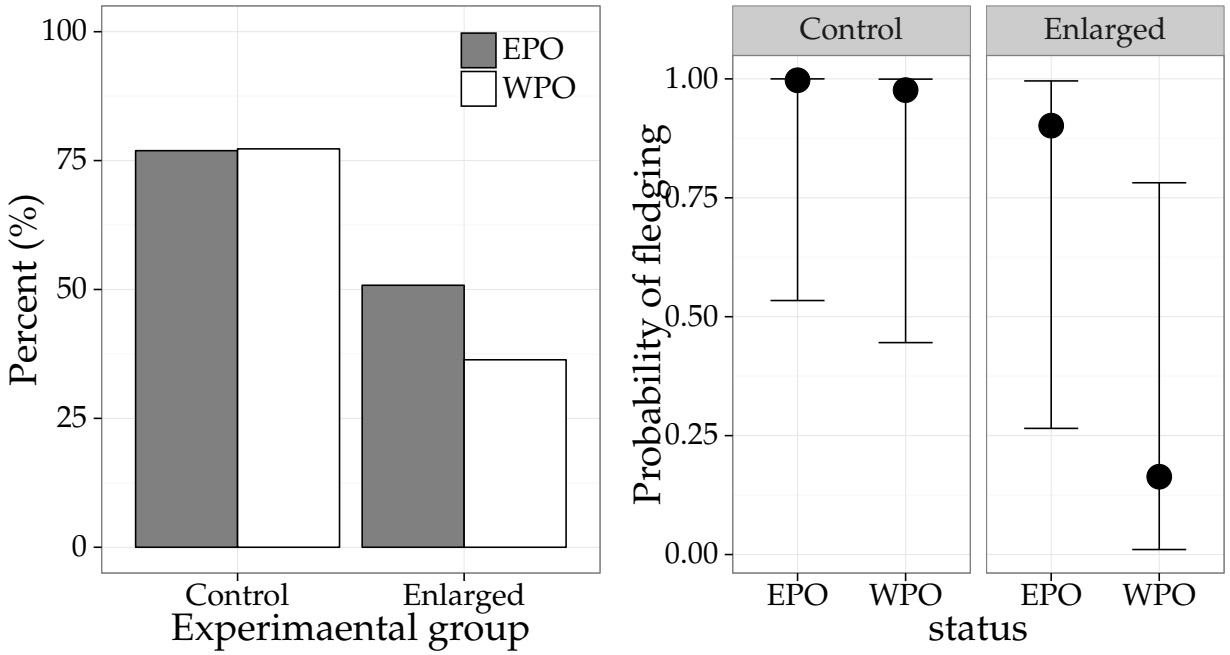

Figure 2.2: The percent of chicks fledged (left panel) and the probability of fledging (right panel) of within-pair offspring (WPO) and extra-pair offspring (EPO) in control and enlarged broods. The left panel plots the percent of WPO (light bars) and EPO (dark bars) chicks that fledged from control (left) and enlarged (right) broods. The right panel shows an effects plot from a binomial GLMM with the probability of fledging as a response. Dots are the estimates and lines are the 95 % confidence intervals on those estimates. The interaction term was not found to be important based on AIC model selection. When tested in each group separately, in control broods both EPO and WPO have the same probability of fledging ($\chi^2_1 = 2.30$, $p = 0.13$), but EPO have a higher probability than WPO in enlarged broods ($\chi^2_1 = 8.75$, $p = 0.003$).

stressful environment for the chicks—chicks in enlarged broods were smaller, had a lower probability of fledging and had shorter telomeres than chicks in control broods (Belmaker 2016, chapter 1). However, our results do not show an effect of EP status on TL or on any of the other measurements (mass, wing length or head-plus-bill length), either in control or enlarged broods. EP status was an important predictor of the probability of fledging, however, in enlarged broods but not in control broods (Fig. 2.2). This pattern for the probability of fledging supports the idea that EPO are higher quality than WPO—EPO had a higher probability of fledging—and that this advantage is context-dependent—the EP status effect is driven by WP chicks in enlarged broods.

So, why do we find an effect of EP status only for the probability of fledging and not any of our other metrics? First, chicks were only sampled for TL once at the age of 12 days. Chicks that did not survive to that age were not sampled, as the TRF assay is sensitive to DNA degradation (Haussmann and Mauck 2008a). It is possible that short-telomere chicks were overrepresented among these dead chicks, and that this is the cause of the lack of effect on TL we observed—only high quality, long-telomere chicks survive, who can overcome the stress of the manipulation. As we do not have data on the chicks that died before measurement we cannot rule out this possibility. However, looking at the TL effect on survival from day 12 onward reveals that short-telomere chicks are not less likely to fledge than are long-telomere chicks (Belmaker 2016, chapter 1). While this does not prove that the results are not biased by selective mortality of short-telomere chicks, it provides some evidence that the results are not biased in this way.

Second, it is possible that the chicks were sampled too early in development to show an effect. The interaction of experimental group and EP status did not have a significant effect on TL, but figure 2.1 shows a suggestive decrease in the TL of WPO in enlarged broods. The telomere samples in this study were taken when the chicks were 12 days old to minimize the risk of premature fledging. However, Tree Swallow chicks fledge at 21 days of age. This means the chicks had a lot more time to experience the manipulation after we took the sample. It is possible that, had we measured the chicks closer to fledging, we would have found a stronger effect of EP status on TL. TL in 12-day-old Tree Swallow chicks is more strongly affected by heritable factors than the environment (Belmaker 2016, chapter 1). As the chicks mature, both environmental effects and the action of genes associated with TL (telomerase, the enzyme responsible for lengthening telomeres, genes related to stress, etc.) will determine a larger proportion of TL variation; and it is possible that EPO have a better version of this telomere-maintenance system. In this case, the advantage to EPO will only be evident after this telomere-maintenance

system has had a chance to operate. The activity of the telomere-maintenance system can be estimated by measuring how fast telomeres shorten. Telomere shortening rate has also been shown to affect survival, independently of telomere length (Bize et al. 2009; Epel et al. 2009; Salomons et al. 2009).

The proper metric in our case might indeed be telomere-shortening rate rather than TL, however, as we only have one telomere sample per chick, we have no way of testing this hypothesis. The proxy and context chosen will ultimately determine if a given study can or cannot detect the hypothesized effect, but choosing them is not an easy task—there is no a-priori way of knowing whether a given metric and context are appropriate. If this is true, then some studies, which happen to choose an appropriate fitness proxy and context, will find a benefit to female EPC while others will not. Here, when we choose the proper metric (probability of fledging) we see an effect not visible with others (TL and size). Previous studies to report a context-dependent benefit to EP status used various metrics as a proxy for fitness, and various contexts to test the effect of EP status on the chosen proxy (Reviewed in Schmoll 2011).

Choosing the appropriate traits in the EP sire is difficult for the female as well. If by engaging in EPC a female only seeks to diversify her brood (Westneat et al. 1990; Williams 1975) there is no problem of mate choice, as any additional mates will achieve that goal. Indeed there is evidence that in Tree Swallow the purpose of female EPC is a diverse brood (Dunn et al. 2009; Kempenaers et al. 1999; Stapleton et al. 2007; Whittingham and Dunn 2010; Whittingham et al. 2006). However, there is also evidence that female Tree Swallows choose a higher-quality EP sire (Bitton et al. 2007; Kempenaers et al. 2001; O'Brien and Dawson 2007; Whittingham and Dunn 2014; Whittingham and Dunn 2016). In the latter case, the traits that the EP sire is likely to pass on are paramount, but how does a female choose an EP sire?

'Good genes' hypotheses for female EPC in general state that EPO benefit from inheriting advantageous alleles from the EP sire (Griffith et al. 2002). However, what defines a "good gene" is context-dependent (Schmoll 2011). The context in which the benefit of an allele is measured can be that of the external environment or that of the internal genetic environment with which the allele must interact (Schmoll 2011). Who the best EP sire is will depend on the environment the chicks are likely to find themselves in in the future, and on the alleles that sire will pass on. However, at the time the female is choosing EP sires, both the environment in which those 'good genes' will be evaluated and what alleles the chicks will inherit from their EP father are unknown to the female (Hasson and Stone 2010). The female has to resort to choosing an EP sire based on any number of sexual signals (e.g., Whittingham and Dunn 2016), that only *correlate* with fitness *on average*. So, by the same token, a female's mate choice does not always show a benefit (Schmoll 2011). Indeed, the genetic benefits from mate choice are likely small (Arnqvist and Kirkpatrick 2005; Kirkpatrick and Barton 1997; Moller and Alatalo 1999). Our ability to detect a small effect depends on our sample size, and most field studies suffer from low sample sizes (Schmoll 2011). Alternately, we could follow a population over many generations but, as it is difficult to do, this is rare. However, the few studies that use a long-term data set still do not find evidence for the good-genes hypothesis (Hsu et al. 2014; Lubjuhn et al. 1999; Schmoll et al. 2003), perhaps because in those systems there is no such benefit (Forstmeier et al. 2014). Lastly, the ability of females to choose the best mate as an EP partner is limited by the availability of mates, the information she has and many other random factors. For example, some females might benefit from a high quality EP mate but not be able to find one. This will add noise to the data further obscuring our ability to detect any benefit to EP behavior. Together, the uncertainty in choosing a high-quality mate, the small genetic benefits from mate choice and the limited ability of females to choose a high-quality EP mates, mean that, while in the long run, on average, choosing a high-quality mate as an EP sire is advantageous, in the short-term,

on a per breeding season basis, that advantage is not always seen—even if a benefit to female EPC is present we might lack the ability to detect it.

To summarize, EPO in this study had a higher probability of fledging than WPO but only in enlarged broods. We could not detect an advantage to EPO in TL or size, maybe because we sampled too early, these are not the proper metrics or context, or we lack the ability to detect such benefits.

CHAPTER 3

PATTERNS OF CO-VARIATION OF TELOMERE LENGTH, CONDITION, LIFE HISTORY AND FITNESS IN A SHORT-LIVED BIRD SPECIES

Abstract

Telomeres are the long repetitive sequences capping and protecting the ends of chromosomes. Telomere length (TL) has been shown to be important in many aspects of biology including life history, behavior, physiology and aging. Short TL is associated with poor health, survival, reproductive success and fitness in several species. Most studies that associate TL with survival and reproduction have been conducted on long-lived species. However, predictions about the association between telomere length, quality and fitness might be different for short-lived species, which invest energy differently than long-lived ones. Here, we followed a population of Tree Swallows, *Tachycineta bicolor*, a short-lived passerine, for three breeding seasons and measured fitness components, condition, life history traits and telomere length. Males had longer telomeres than did females, body condition was positively related to TL in both sexes of parents, and longer telomeres in females were associated with a smaller proportion of chicks fledged. TL did not shorten detectably with age, and its variation was not related to clutch initiation date, clutch size, the probability of fledging young or adult return rates. These results suggest that telomere length might not be an important fitness-determining factor in Tree Swallows, either because they are short-lived or because of their dependence on weather. More studies on species with a broad range of life histories are needed to generalize how telomere length impacts variation in individual reproductive performance and survival.

Introduction

In the past decade there has been a growing understanding of the importance of telomeres to many facets of animal biology such as aging, physiology, behavior and life history (Haussmann and Marchetto 2010). Telomeres are long repetitive sequences of DNA that cap and protect the ends of chromosomes (Harley 1991). Telomeres serve two main functions: first, they prevent the DNA repair machinery from identifying the ends of chromosomes as breaks in the double-stranded helix (Nugent et al. 1998); second, they prevent the loss of important genetic information due to the normal shortening of the chromosome with each cell replication (Levy et al. 1992). Telomeres are thus very important for the maintenance of chromosomal stability and normal cellular function.

Telomeres shorten with each replication (Levy et al. 1992), and critically short telomeres lose functionality, which starts a cascade that can lead to reduced organismal function and mortality (Blasco 2005; Campisi 2005). Short telomere length (TL) has been shown to be an important predictor of bad health and is associated with many cancer types and many age-related diseases in humans (Blasco 2005). Because of the importance of telomeres to human health, most studies on telomeres focus on humans and mice and, indeed, most of what we know about telomere dynamics comes from model organisms. The interest in telomere biology in non-model organisms has begun to rise only recently but is growing rapidly. Studies on wild populations show that short TL is associated with low adult survival rates (Angelier et al. 2013; Bize et al. 2009; Haussmann et al. 2005; Heidinger et al. 2012; Salomons et al. 2009; Stier et al. 2014; Verhulst et al. 2006); that individuals with longer telomeres have higher reproductive success (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012); that individuals with different TL forage differently (Young et al. 2015) and make decisions differently (Bateson et al. 2015). Not only are we

learning a great deal about the effects that TL variation can have on the biology of organisms, but we are also learning much more about how biology feeds back onto variation in TL shortening rates. Early-life stress can cause TL to decline more rapidly (Entringer et al. 2011; Epel et al. 2004; Geiger et al. 2012; Haussmann et al. 2011; Kotrschal et al. 2007), as can disease (Asghar et al. 2015a) and reproductive effort (Bauch et al. 2013; Reichert et al. 2014b; Sudyka et al. 2014).

A causal relationship between TL, health and performance has been implicitly assumed in the literature and, while plausible, it is still unclear whether TL actually acts as a causal agent or is merely correlated with reduced fitness (Simons 2015; but see Reichert et al. 2014a). Telomeres are a part of an interacting physiological network that affects the survival of an individual and its ability to reproduce (Haussmann and Marchetto 2010). While acknowledging the complexity of causes affecting TL and its associated biological effects, TL provides us with a metric—telomeres can be short or long—that can be used as a relatively easily interpreted *marker* for the state of the entire network (Bateson 2016). With this marker as an indicator, we can start to ask how the network is affected by environmental variables, and in turn, how it affects the behavior and life history of the individual. Although the detailed causality of the effect of TL on fitness has not yet been determined (Simons 2015), TL has been used extensively as a proxy for quality (Bateson 2016; Bauch et al. 2013; Le Vaillant et al. 2015). However, TL can be associated with reproductive success positively (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012), negatively (Bauch et al. 2013) or they can have no relationship (Lopez-Sarasa 2015). This variation in the correlates of TL variation might arise because of variation in individual quality that might be masking other important factors, that potentially affect the association between TL and reproduction (Bowlin and Winkler 2004; Partridge and Harvey 1988; Smith 1981).

Despite the wide interest in TL biology, most studies to use TL have been done on long-lived species (Sudyka et al. 2015). Studying short-lived species is important because long and short-lived species trade-off energetic investment differently—short-lived species invest more energy in current reproduction while long-lived ones save for future reproduction (e.g., Promislow and Harvey 1990)—and the selection pressures determining their fitness are different as well (MacArthur and Wilson 1967; Newton 1989). It stands to reason that the role of the individual's physiology (with TL as a marker) in determining fitness might be different between them as well (Crossin et al. 2016). Studies have confirmed that telomeres of short-lived species (mean life expectancy of less than six years) shorten at a faster rate than do those of longer-lived species (Haussmann et al. 2003; Sudyka et al. 2015), but relatively little has been done to link the TL of short-lived species to fitness. Attempting to make this link between TL and fitness in short-lived species will help evaluate the generality of using TL as a marker for quality.

While several studies have shown the TL of short-lived species is a cost of increased investment (Asghar et al. 2015a; Reichert et al. 2014b; Sudyka et al. 2014), and predicts the probability of survival (Angelier et al. 2013; Barrett et al. 2013; Haussmann et al. 2005; Heidinger et al. 2012; Pauliny et al. 2006) and reproductive success (Pauliny et al. 2006), most of these studies have been conducted in captivity (Heidinger et al. 2012; Reichert et al. 2014b). Captive studies intentionally remove any environmental variation that might be important in determining the fitness of individuals, so studying telomere dynamics in the wild is crucial. A few studies on short-lived species report that variation in TL predicts mortality rate (Angelier et al. 2013; Barrett et al. 2013; Haussmann et al. 2005), but to our knowledge only one study in the wild on a short-lived species reported that variation in TL predicts variation in reproductive success (Pauliny et al. 2006). An individual's fitness is determined both by its survival and its reproductive output. If we are to study what role telomeres play in the life history of species and how general that

role is, it is vital that we study the relationship of TL to both survival and reproductive success in short-lived species as well.

To assess the relationship between TL, quality and fitness of individuals in a short-lived species, we followed a population of Tree Swallows (Fig. 3.1), a small, short-lived passerine, through three breeding seasons. We measured and correlated metrics of condition, survival and reproductive success. In a cross-sectional study of Tree Swallows, TL was found to shorten with age (Haussmann et al. 2003), and in a separate study, the expression of telomerase, the enzyme responsible for telomere elongation, was found to be high in hatchlings but dropped sharply in adults (Haussmann et al. 2004; Haussmann et al. 2007). Greater TL in Tree Swallows has also been associated with higher adult return rates (Haussmann et al. 2005), and males and females with longer TL raised lighter nestlings (Ouyang et al. 2016). Of the studies cited above, three were conducted in a Tree Swallow population ~17 km away from the current study site (Haussmann et al. 2003; Haussmann et al. 2005; Haussmann et al. 2004). Given the scarcity of studies on short-lived species that report on both survival and reproductive success, and this foundation of studies on Tree Swallow telomeres, more research into further aspects of their dynamics and biological effects seem especially worthwhile.

Figure 3.1: *Tachycineta bicolor* (Tree Swallow), a small, short-lived, aerial insectivore has become a popular model system for a variety of fields. Photo by David W. Winkler.

Methods

Study system

During the breeding seasons of 2012-2014 we followed the nesting attempts of a total 347 individual *Tachycineta bicolor* (125, 108 and 114 per year, respectively) breeding near Harford, NY (42.44°N, 76.23°W). As females are easier to catch than males we caught 263 females and only 84 males. Tree Swallows are small, migratory, aerial insectivores, with an average life span of 2.7 years (Butler 1988). They are common breeding birds in North America and have been used as a model system for studies ranging from life history and behavior to physiology (Jones 2003). The study site is a cattle grazing ground with 130 nest boxes mounted on fence posts, ~1.8 m above the ground and at a distance of ~20 m between adjacent boxes. Annual occupancy of the nest boxes was ~70 %.

Nests were monitored daily to determine the day the first egg was laid (clutch initiation date). Clutch completion was set as the day the last egg was laid. During incubation, boxes were monitored every third day, and females were captured in the box only after the seventh day of incubation to reduce the risk of abandonment. Once captured, every bird was measured for body mass, head-plus-bill length and wing length with methods and standards adopted by the 'Golondrinas de las Americas' project (`http://golondrinas.cornell.edu`). In addition, the following body condition metrics were measured: 1. Pectoral score, a 4-category measure of the size of the breast muscle, with a concave muscle with a visible keel getting a measure of zero and a fully convex muscle a measure of three; 2. breast muscle thickness measured with a model USL 48 portable ultrasonic fault-detector machine (Krautkramer-Branson ultrasound, Lewiston, PA; See Winkler and Allen 1996); 3. two size-controlled mass measures were calculated: mass divided by wing length and mass divided by head-plus-bill length; and lastly 4. the amount of sub-cutaneous fat in four ventral areas was scored: abdominal (Percent cover of the area between the ribs and the cloaca on a 5-category scale), transverse (Width in mm of the narrowest point of the fat along the bottom edge of the ribcage), lateral (Percent cover of the area between the wing and the body on a 4-category scale), and furcular (A 4-category score of the amount of fat in the hollow between the furcula and the neck).

there are many possible measures of body condition, each with advantages and disadvantages (Brown 1996). We chose these measures because they were easily obtained without the need to euthanize the bird, allowing us to follow the nesting cycle to completion. However, all these condition metrics were correlated (Table 3.1), and we therefore combined all of these measures into one index of condition using a non-linear principle component analysis (NLPCA). This dimension-reduction was conducted using the 'homals' package (version 1.0-6; De Leeuw and Mair 2009) for R (R Core Team 2015). A NLPCA was needed because many of these metrics are ordinal, violating the assumptions

of a traditional linear PCA. The correlations between the variables and the loadings from this NLPCA are summarized in table 3.1. All of the variables loaded similarly on the first principle component (PC1) and PC1 explained 70 % of the variation. To account for adult changes in body condition throughout the season we used the residuals from a regression of their condition index on days pre- and post-hatch. We used a linear regression for this correction as an additive smooth did not fit better. As females and males have different dynamics of change in body condition with respect to hatching day (Boyle et al. 2012), we did this correction separately for each sex.

For each adult captured we noted its age. Tree Swallow females show delayed plumage maturation, in which second-year (SY) birds (which are in their first nesting season as a breeder) have a brown plumage, and older birds show the characteristic iridescent blue plumage (Hussell 1983). This allows us to estimate with 95 % accuracy the age of breeding females that were first encountered on the site as un-banded, brown-plumaged birds (Hussell 1983). In addition, a few individuals that fledged from our site (all of which were banded as nestlings) returned to breed there, and banding records allowed us to measure their precise age. In cases where this was not possible, we noted the minimum age of each individual. Males, who do not show delayed plumage maturation, were aged as follows: each un-banded male was assigned a minimum age of one year. Each subsequent year that male was encountered we increased the estimate by one. Males that were banded on our site as nestlings and returned were assigned their true age.

Lastly, a blood sample was taken from the brachial vein for telomere length analysis. Between 20 to 150 µl were taken using a heparinized micro capillary tube. Half of the blood was put into lysis buffer for genotyping and the other half was put into an empty 1.5 ml micro-centrifuge tube and stored on ice until further processing in the lab.

Table 3.1: The correlations between Mass, pectoral score (PS), four fat scores: abdominal (AB), transverse (TR), lateral (LA) and furcular (FU), breast-muscle thickness (BMT) and two size controlled mass metrics: mass divided by wing length (SCM1) and mass divided by head-plus-bill length (SCM2). These metrics were combined using a non-linear principle component analysis and the first principle component (PC1) was used as the condition index. The loading of each metric on PC1 are in the grey row. PC1 explained 70 % of the variation.

	Mass	PS	AB	TR	LA	FU	BMT	SCM1	SCM2
PC1 loadings	0.31	0.26	0.28	0.26	0.28	0.27	0.22	0.31	0.31
Mass	1								
PS	0.61	1							
AB	0.69	0.59	1						
TR	0.61	0.58	0.71	1					
LA	0.71	0.63	0.74	0.62	1				
FU	0.71	0.54	0.67	0.57	0.68	1			
BMT	0.57	0.56	0.47	0.41	0.44	0.45	1		
SCM1	0.96	0.66	0.72	0.64	0.74	0.72	0.59	1	
SCM2	0.95	0.68	0.74	0.66	0.75	0.73	0.58	0.94	1

At the end of the day, telomere samples were spun down at 3500 rpm for 5 minutes and the plasma was removed. One ml of NBS buffer (90 % new-born calf serum and 10 % DMSO) was added and mixed with the red blood cells (RBCs). The samples were then frozen slowly and kept at −80°C for storage until analysis.

After capture, the breeding attempts of all measured birds were followed to completion. We monitored clutch size, whether the attempt was successful and how many chicks fledged. In the subsequent year we noted which birds returned to breed again as a proxy for survival. Although it is impossible to distinguish true survival from dispersal, return rates are often used as a proxy for survival (e.g., Angelier et al. 2013).

Telomere length analysis

Telomeres were measured using the TRF assay (Kimura et al. 2010). A full description of the protocols used are described in Belmaker (2016, chapter 1). In short, DNA was extracted with a Gentra Purgene extraction kit for the extraction of high quality, high yield DNA (Qiagen, Hilden, Germany), using an isopropanol-ethanol extraction. DNA integrity was checked on a 0.8 % agarose gel made with $1x$ TAE run for 1 hour in 120 V. Ten µg of DNA were digested for at least 16 hours at 37 °C with a combination of three restriction enzymes (RsaI, HaeIII and HinfI) and run on a 0.8 % agarose gel in a pulsed-field gel electrophoresis rig for 19 hours ($3\,\mathrm{V\,cm^{-1}}$, 0.5 s initial switch time and 7 s final switch time) alongside three lanes of 1 kb extension ladder from Invitrogen and two standard lanes made of either Domestic Chicken blood or Tree Swallow blood. The gel was then dried and hybridized overnight with a radioactive probe ('CCCTAA' x 4), placed on a phosphor screen (Amersham Bio-sciences, Buckinghamshire, UK) for at least two days and visualized using a Storm 540 Variable Mode Imager (Amersham Biosciences).

This procedure results in a TL distribution, per sample (Kimura et al. 2010). From each distribution we measured mean TL, skew, kurtosis and the 10th to 90th deciles. All these metrics were combined using a principle component analysis into one measure of TL. The rationale for this reduced dimensionality of the telomere measures is described in Belmaker (2016, chapter 1).

Statistical analysis

All analyses were carried out in R (version 3.0.2; R Core Team 2015). We used linear and generalized linear mixed-effect models with the 'lmer' and 'glmer' functions from the 'lme4' package (version 1.1-11; Bates et al. 2013). We scaled continuous variables to aid in parameter estimation. Some nesting attempts were manipulated for a different experiment. The individuals involved in these manipulations were only used in regressions of variables manifested before the manipulation (clutch initiation date, clutch size etc.) but not for variables that might have been affected by the manipulation, like fledging success. To increase sample sizes we initially included cases where the same bird was caught in successive years and added bird ID as a random effect. However, this approach resulted in a residual plot indicating bias—the plot of the residuals over fitted values showed a distinct pattern. To solve this problem we only used each individual bird once. Model validation plots after this improvement showed no indication of bias. This procedure removed 69 females 13 males and 194 females and 71 males were left in the final analysis. In these analyses we only used the first breeding attempt per season of each bird.

To test how individual traits correlated with TL we constructed a model with TL as a response variable. As predictors we added wing length, head-plus-bill length and the condition index, corrected for days before the brood's hatching, and parental age (either minimum age or true age; see below). We also included the interaction term of sex and each of the predictors. To test how breeding success and return rates vary with TL we used either clutch initiation date, clutch size, the probability of fledging at least one chick, the proportion of chicks fledged or return rates as a predictor variable. As fixed effect we used TL, year and age. All models were simplified using a stepwise selection procedure using AIC as the criterion for exclusion or inclusion. In every step we dropped terms sequentially and the term that most improved the AIC by being excluded from the model

was removed. We then tested the addition of previously excluded terms. The final model was determined as the one where no other exclusion or addition of terms could further improve the model AIC. P-values for the final model were obtained using likelihood-ratio tests.

As telomeres shorten with age (Haussmann and Marchetto 2010) it is important to control for age in our models. For all birds we have an estimate of their minimum age, and only for a subset of those do we have their exact age. Each model was run twice, once with minimum age and once with true-age as a predictor. We report the results from both models but only in one case (wing length) did the results differ.

As males rarely enter the box during incubation, they are harder to catch than are females, and sample sizes for males are much lower than those for females. When testing the correlation between TL and attributes of the individual we could use all the males we caught and sex was added as a fixed effect in the models. However, because the vast majority of males that were caught were part of a brood manipulation we could not use them in models for nesting success and return rates. In those models we only used females that were not part of the manipulation, and males were excluded. Clutch initiation date and clutch size were considered female traits, and males were excluded from the analyses of those variables as well.

In models testing the association between TL and individual traits, year was added as a random effect. Running the analysis separately by year was impossible due to model convergence problems. For models of breeding success and return rates year was added as a fixed effect to control for any yearly variation in these variables.

Results

The correlation between TL and parental age, size, sex and condition

We tested the correlation between TL as a response variable and parental age, size, sex and body condition as predictors. The model was run once using the known-age of each bird and a second time using the minimum-age. Known age interacted with sex to affect TL, such that in females TL did not change with age but TL shortened with age in males (Table 3.2). However, when the lone data point for a know-aged male older than three years (six years old) was removed form the model, neither age nor its interaction with sex appeared as important predictors of TL (Table 3.2). In the minimum-age data, the range of ages was more fully sampled so there was no need to remove any data points. In this case, the interaction of sex and age remained in the model but the relationship is not strong, as evidenced by the low ΔAIC and borderline p-value (Table 3.2).

Table 3.2: The results from the final three models with telomere length (TL) as a response: the known-age model run with all data points (Known age), the known-age model with one influential datum removed (Known age 2) and the minimum-age model (Minimum age). The ΔAIC is the differnce in AIC value between the final model and the model with that term dropped. P-values were obtained using likelihood ratio tests. Cells with no entry mean that term was not included in the final model or that it does not have an independent effect on TL.

	Known age			Known age 2			Minimum age		
Predictor	ΔAIC	χ^2_1	P	ΔAIC	χ^2_1	P	ΔAIC	χ^2_1	P
Sex	-5.12	7.12	0.008	-2.75	4.75	0.029	-8.09	10.09	0.001
Age									
Wing length							-0.61	2.61	0.106
Condition	-0.71	2.71	0.1	-1.93	3.93	0.048	-0.99	2.99	0.084
Sex *x* Age	-0.78	2.78	0.096				-0.84	2.84	0.092

The effects of age in this model were assessed between individuals. Because, se-

lective mortality of short-telomere individuals can mask TL shortening with age (Haussmann and Mauck 2008b), we also ran a longitudinal model with all the birds that were captured more than once. This longitudinal model also did not show TL to shorten with either known-age (LMM: $\beta = -0.25 \pm 0.35$, $\chi^2_1 = 0.49$, $p = 0.48$) or minimum-age (LMM: $\beta = 0.36 \pm 0.28$, $\chi^2_1 = 1.05$, $p = 0.31$).

Sex consistently appeared in the final model as an important predictor of TL, with males having longer telomeres than females (Fig. 3.2). The condition index, corrected for days before hatching, was positively correlated with TL using both known-age and true-age (Fig. 3.3), but its interaction with sex was not included in the final model. Wing length was negatively correlated with TL but only when controlling for minimum-age (Table 3.2).

Fitness

Female TL did not predict clutch initiation date (LM: $\beta = -0.15 \pm 0.14$, $\chi^2_1 = 1.14$, $p = 0.28$), clutch size (GLMM with Poisson family: $\beta = 1.7 \times 10^{-4} \pm 0.01$, $\chi^2_1 = 0.03$, $p = 0.87$), the probability of fledging at least one chick (GLMM with binomial family: $\beta = 0.04 \pm 0.08$, $\chi^2_1 = 0.30$, $p = 0.58$) or return rates (GLMM with binomial family: $\beta = 0.03 \pm 0.06$, $\chi^2_1 = 0.24$, $p = 0.62$). Females with longer telomeres fledged a smaller proportion of chicks in both the minimum-age and the known-age model (GLMM with binomial family: $\beta = -0.07 \pm 0.03$, $\chi^2_1 = 4.19$, $p = 0.04$; Fig. 3.4).

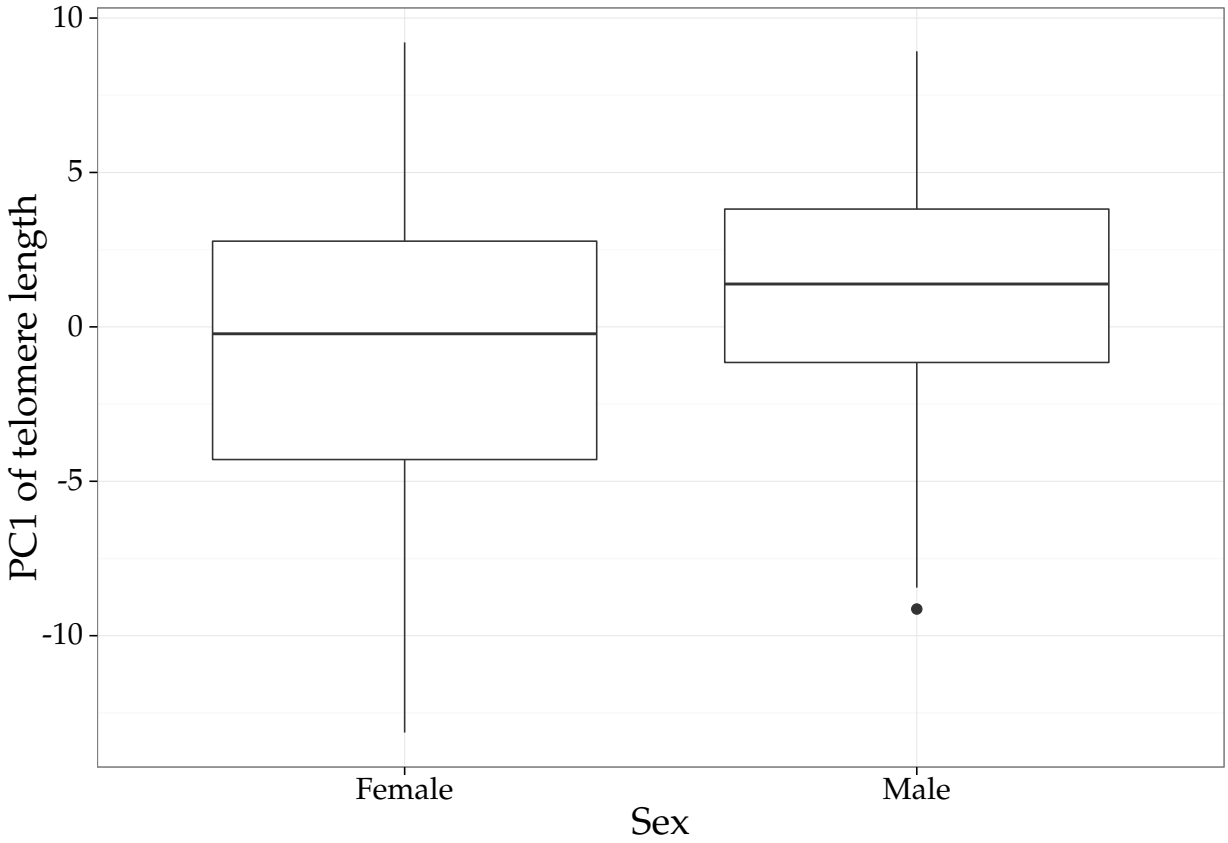

Figure 3.2: Differences in telomere length (TL) between males (right) and females (left) of adult Tree Swallows. Three models were run: one with the known age of each bird, one with known age but with one influential datum removed and one controlling for minimum age. Sex was the only consistent variable to reliably predict TL with p-values of 0.008, 0.029 and 0.001 respectively. The dot represents one outlier.

Discussion

This study evaluated the role of variation in telomere length on Tree Swallow survival and reproductive success. The most robust finding is that males had longer telomeres than did females, irrespective of age. A sex difference in TL has been reported in many species, from a variety of taxa, but sex differences in TL are rare in birds (Barrett and Richardson 2011). Most studies to report on the effect of sex show no difference in TL between males and females or show females to have longer telomeres than males (reviewed

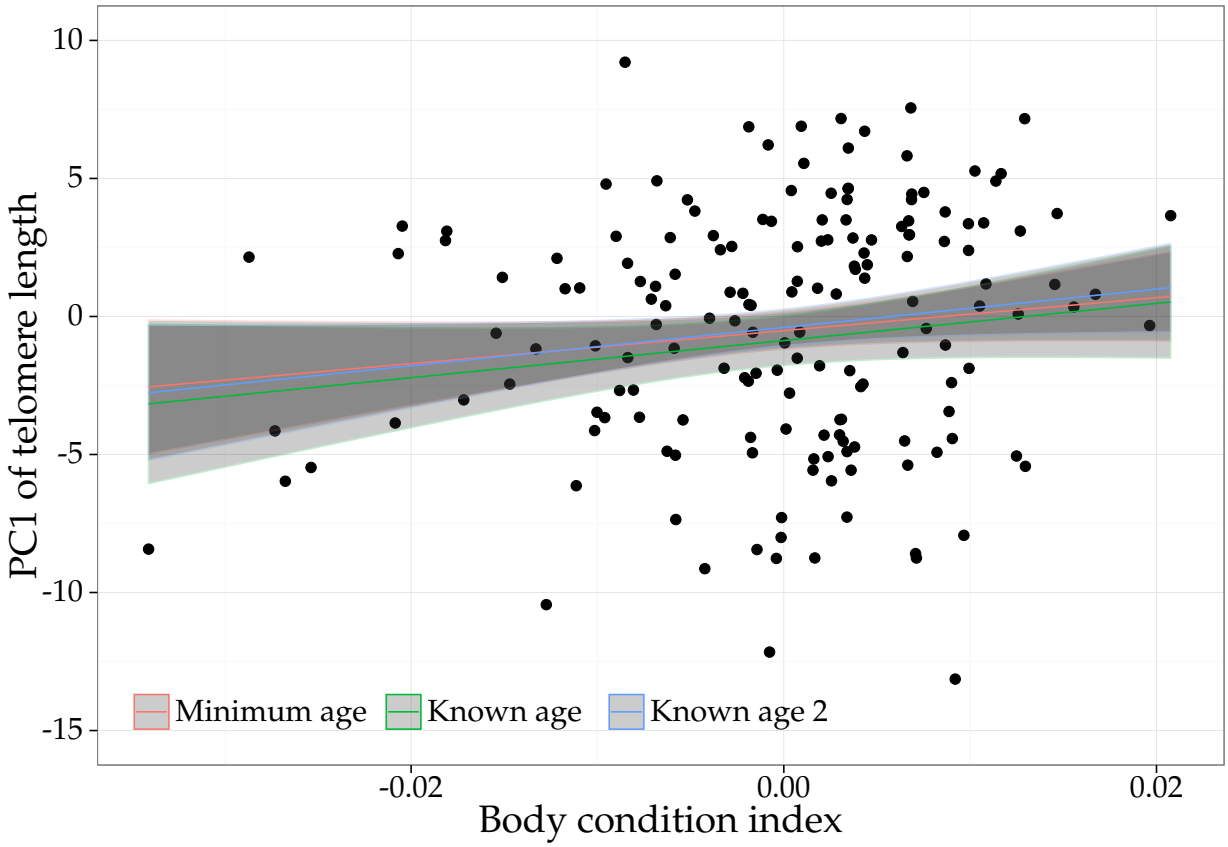

Figure 3.3: The correlation between body condition and telomere length for both sexes. The three regression lines correspond to the three models fit with minimum age (red), known age with all data points (green) and known age with one influential datum removed (blue). The estimates for all three models correspond well and body condition was retained in the final model for all three, though with p-values of borderline significance (0.1, 0.05 and 0.08 respectively).

in Barrett and Richardson 2011). Only a few show females to have shorter telomeres than males (Bebbington et al. 2016; Foote 2008; Foote et al. 2011a; Horn et al. 2011; Pauliny et al. 2012; Young et al. 2013; but see Barrett et al. 2013). Several hypotheses have been proposed to explain this difference, but none can explain all the variation in sex-specific patterns observed among different taxa (reviewed in Barrett and Richardson 2011). First, it is possible that a faster growth rate in males is responsible for this sex-difference (Foote et al. 2011b). As in many other species, male Tree Swallows are larger than females (Winkler et al. 2011) and, because telomeres shorten proportionally to growth rate (Geiger

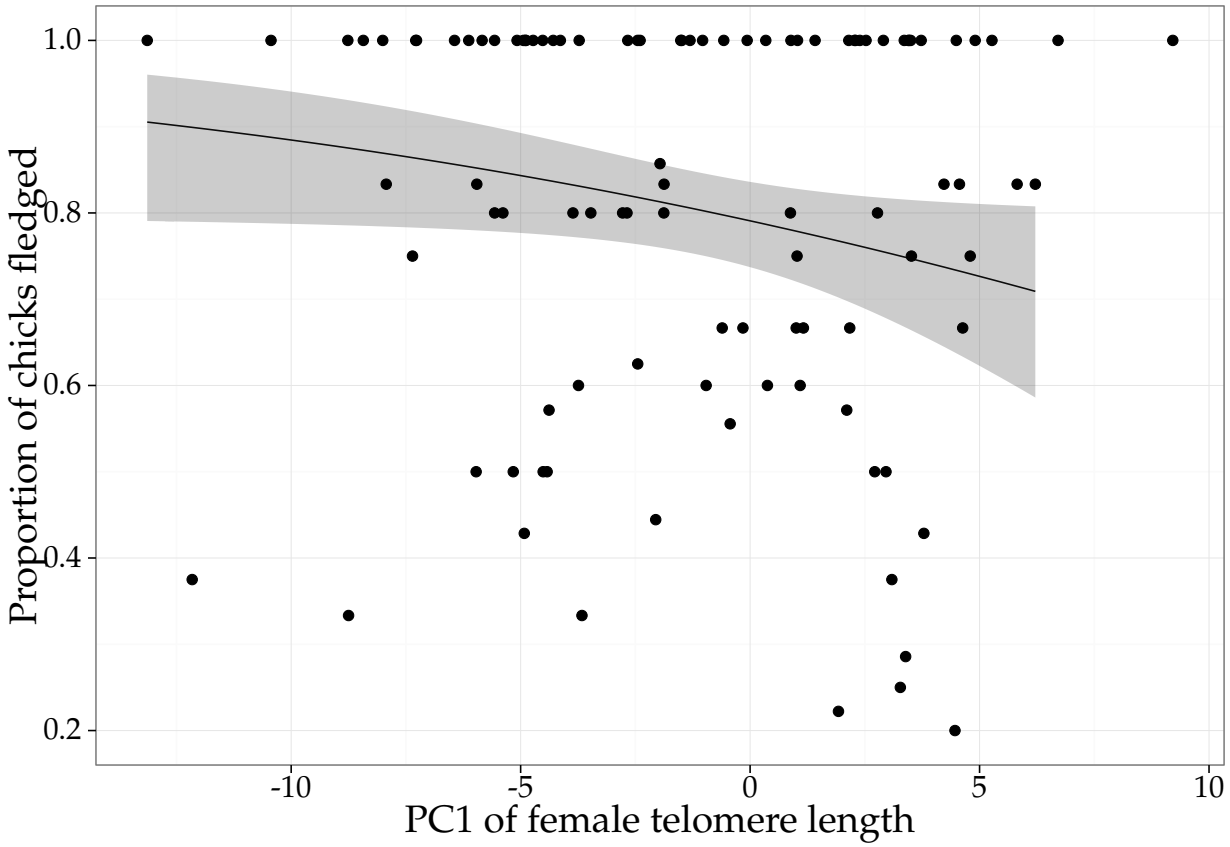

Figure 3.4: The correlation between telomere length of adult females and the proportion of young fledged. Only broods that fledged at least one chick were included in this analysis.

et al. 2012; Reichert et al. 2015b; Scott et al. 2006), we would expect to see a difference in TL between males and females. However, here we report males to have *longer* TL than females, the opposite of the predicted pattern based on growth. Another possibility for the longer telomeres in male Tree Swallows is that females might be working harder during the breeding season than males. Only females lay and incubate eggs, and incubation is costly (Ardia et al. 2010; Ardia and Clotfelter 2007; Ardia et al. 2009; Perez et al. 2008). Provisioning rates in Tree Swallows can vary from equal to females provisioning more than males (Leffelaar and Robertson 1986; Lombardo 1991; McCarty 2002; Quinney 1986; Whittingham et al. 2003; Williams 1988), but males rarely feed more than do females. This increased female energy expenditure might produce stress, which is known

to shorten telomeres (i.e., Epel et al. 2004). If females are expending more energy than males in any given season, this could contribute to faster telomere erosion (Bauch et al. 2013). However, if energy expenditure were indeed the cause of the sexual difference in TL, one would expect to see TL in females shortening at a faster rate than in males. This is not the case in our system, as we see that, in neither sex, did TL change significantly with age. It thus appears that reproductive investment alone cannot be the direct cause of this sex difference. Previous results from our system show that male and female 12-day-old Tree Swallow chicks have the same TL (Belmaker 2016, chapter 1). In general chicks do not show TL to be different between the sexes (Caprioli et al. 2013; Nettle et al. 2015; Parolini et al. 2015; Young et al. 2013; but see Foote et al. 2011b), suggesting the sex difference in TL develops during sexual maturation, before the commencement of breeding, and is maintained thereafter. It has been suggested that heterogamy might be associated with the sex-difference in TL (Horn et al. 2011), because the unguarded sex-chromosome might contain deleterious alleles (Barrett and Richardson 2011). If telomere-maintanence genes reside on the unguarded chromosome it would cause faster telomere erosion in the heterogametic sex (Barrett and Richardson 2011). However, this pattern does not seem to be general, as some studies where females are heterogametic show females to have longer TL (Foote et al. 2011b; Jemielity et al. 2007; Olsson et al. 2011; Ujvari and Madsen 2009) and others show females to have shorter telomeres (Bebbington et al. 2016; Foote 2008; Foote et al. 2011a; Horn et al. 2011; Pauliny et al. 2012; Young et al. 2013). Lastly, sex hormones, which play such a pivotal role in sexual maturation that happens in the first year of life, might somehow affect TL as well. There is evidence that sex hormones activate telomerase, the enzyme responsible for lengthening telomeres (i.e., Bayne and Liu 2005; Calado et al. 2009), and that sex hormones can interact with reactive oxygen species to affect TL (Viña et al. 2005). However, the effect of sex hormones on TL is likely complex, as it appears to be context-specific (Bayne and Liu 2005), and some studies show males to have longer telomeres than do females, while others show the opposite (reviewed in

Barrett and Richardson 2011, figure 1). It appears that more research is needed before we can find general patterns and causes of the sex-differences in TL (Barrett and Richardson 2011).

A few other interesting patterns were found: TL positively correlated with our condition index, both in known-age and minimum-age samples. This suggests long-telomere individuals are in better physical condition (Le Vaillant et al. 2015). Wing length was also found to be a significant predictor of TL in the minimum-age sample, with longer-winged individuals having shorter telomeres, consistent with findings in the literature that TL shortens faster with higher growth rates (Geiger et al. 2012; Reichert et al. 2015b; Scott et al. 2006). Lastly, females with longer telomeres fledged proportionally fewer chicks. Together, these patterns suggest that, while birds with longer telomeres are in better condition, growth (i.e., wing length) and reproductive success (i.e., proportion of young fledged) both come at a cost of telomere erosion (Bauch et al. 2013). The patterns we report here are interesting, but none of them is very robust, and we see only a few significant patterns rather than a complete consistent picture. In many cases the ΔAIC associated with these terms is low (Table 3.2), meaning the models with and without the term are equally informative. In addition, the associated p-values from likelihood ratio tests are only borderline significant (Table 3.2). Lastly, while some variables vary with TL, there is no strong and consistent pattern in any one direction. The fact that TL does not seem to be strongly related to fitness in our system is surprising given that previous studies of diverse taxa have shown a connection between TL and survival (Haussmann et al. 2005; Haussmann and Marchetto 2010) and reproductive success (Bauch et al. 2013; Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012).

One especially intriguing difference between the results from this study and the literature is with regards to return rates. One of the first studies showing a negative

correlation between TL and return rates was done on Tree Swallows at a site only 17 km away from that of the current study (Haussmann et al. 2005). There are a few differences in the design and analysis used: First, this study used birds of all ages while Haussmann et al. studied only SY birds. Second, Haussmann et al. tracked one cohort until all birds disappeared from the study population while here we used a presence/absence mixed-model. Lastly, the sample sizes used by Haussmann et al. are lower than those used here (22 vs. 194 females, 71 of which were SY birds). It seems most likely, however, that the most biologically meaningful differences between the studies were factors, such as the proximity to a food supply that is not affected by cold spring weather (Cayuga Lake, to which the Haussmann et al. population is 20 km closer), the years the studies were conducted, etc. Without controlling for all these factors, it is hard to say what is the cause for this discrepancy. We could not analyze our data the same way as did Haussmann et al., as most of our SY birds were manipulated at some point in their life in a way that might be expected to affect their return rates.

Another notable discrepancy is in the failure of the present study to detect telomere shortening with age. Other studies on Tree Swallows have found that telomeres do erode with increased age (Haussmann et al. 2003), and such shortening is expected from the broader literature (Hall et al. 2004), though not always found (Pauliny et al. 2012). Here we do not see that pattern either in a cross-sectional analysis or a longitudinal model. When using minimum-age as a predictor we find TL in females to *increase* slightly with age, and decrease with age in males, but this relationship is only borderline significant. The lack of an age effect on TL might be caused by selective mortality of short-telomered individuals (Haussmann and Mauck 2008b) but two findings cast doubt on this possibility: First, when looking at change in TL within the same individual we still did not find any change in TL with age, either when using known-age or minimum-age; Second, we did not find any effect of TL on return rates. If selective mortality explained

the lack of telomere shortening with age we would expect to see short-telomered individuals disappear from the population at a higher rate than long-telomered individuals. Previous studies on Tree Swallows used a cross-sectional design and estimated telomere shortening rate as 0.391 kilobase pairs per year (Haussmann et al. 2003). Mean TL in our data ranges from 5.7 to 11.1 kilobase pairs, so it is possible that we were not able to detect such a small annual decrease because the variability in TL in our data is so high. Maybe with a larger sample size we would have been able to detect a decrease in TL with age. However, the sample size we used in our study is larger than that used by Haussmann et al. (2003) so we did not have a problem with sufficient power. Our finding that TL does not change with age *is* consistent with findings in the literature that most telomere shortening happens early in life and little shortening happens in adults (Foote et al. 2011b; Hall et al. 2004; Zeichner et al. 1999), but it is hard to explain why this study reports different results than those in Haussmann et al. (2003).

Patterns of covariation between TL and age, condition and fitness do not seem to be general across all study systems. For example some studies find a negative relationship between TL and reproductive success (Bauch et al. 2013), others find a positive one (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012) and some no relationship (present study; Lopez-Sarasa 2015). Similarly, some studies find chick TL to predict survival (Heidinger et al. 2012) while others do not (Caprioli et al. 2013). Viewed in this light, the differences between this study and those of Haussmann et al. (2003) and Haussmann et al. (2005) are just a striking example of the general variability of empirical TL-related research.

There are a few potential reasons why the role TL plays in determining fitness is study-specific. First, we cannot rule out the possibility of laboratory or other error in the data, but we do not think it is likely, as all the metrics we measured are easily obtained

with relatively high accuracy, and our sample sizes are in general high. Only one observer (AB) made all the measurements to avoid bias, and lab work was all done in the same lab, that of the same Mark Haussmann, author of the earlier Tree Swallow telomere papers. The coefficients of variation in our measurements of TL, based on standard samples run twice on each gel, is 9 % and 5 % for the two standards used, which is within the range reported in the literature. Detected associations in our data are weak, and stronger correlations would have been detected despite the noise. Regardless, the possibility of biases in lab techniques used does exist (Horn et al. 2010). The study of telomeres in ecology and evolution is relatively new and there is still much heterogeneity in techniques (Nussey et al. 2014). While we do not believe that the entire variation in observed patterns can be attributed to TL measurement methods it is important to be aware of such potential sources of variation (Nussey et al. 2014).

Another possibility is that TL is not an important predictor of fitness in short-lived species. The Tree Swallow is short-lived with an average life span of ~2.7 years (Butler 1988), while most studies have been done on long-lived species (Sudyka et al. 2015). Short-lived species invest more energy in current reproduction while long-lived ones save for future reproduction (e.g., Promislow and Harvey 1990), and the selection pressures determining their fitness are different as well (MacArthur and Wilson 1967; Newton 1989). It stands to reason that the role of the individual's physiology (with TL as a marker) in determining fitness might be different between them as well (Crossin et al. 2016). Studies have confirmed that telomeres of short-lived species (mean life expectancy of less than six year) shorten at a faster rate than do those of longer-lived species (Haussmann et al. 2003; Sudyka et al. 2015). It is possible that this difference in how short- and long-lived species maintain their telomeres can explain why we do not find TL to be a good predictor of fitness in our system. Other studies conducted on short-lived species do find a correlation between TL, survival and reproductive success (Angelier et al. 2013;

Asghar et al. 2015a; Haussmann et al. 2005; Heidinger et al. 2012; Pauliny et al. 2006), but most of the studies on short-lived species have been conducted in captivity (i.e., Heidinger et al. 2012). Captive studies remove many of the potentially important variables that determine fitness. Weather, which so strongly affects the reproductive performance of Tree Swallows (Winkler et al. 2013), is a notable example. It is possible that, while the physiological mechanisms that shape TL might still be the same in short- and long-lived species (Sudyka et al. 2015), the fitness of the individual might be more affected by other factors than by these internal physiological mechanisms. When conducting captive studies, and removing environmental effects, we are allowing the physiological fitness-determining mechanisms to be more pronounced than they would be in a natural setting. However, in contrast to the results we present here the few studies to use short-lived species in the wild do find TL to be a good predictor of survival (Angelier et al. 2013; Barrett et al. 2013; Haussmann et al. 2005). A connection between TL and reproductive success in short-lived species is much more scarce. In dunlins, *Calidris alpina*, the number of recruits in a male's lifetime was higher for long-telomere birds than for short-telomere birds but this relationship was not quite significant (Pauliny et al. 2006). Long-lived species also vary in this respect, and the relationship between TL and reproductive success can be positive (Le Vaillant et al. 2015; Plot et al. 2012), negative (Bauch et al. 2013) or flat (Lopez-Sarasa 2015).

Another possibility is that the relationship between TL and fitness can be dependent on the specific life history of the species under study. The Tree Swallow is an aerial insectivore and as such is very susceptible to short-term weather fluctuations. When temperature drops below a certain threshold the insects do not fly and the adults cannot feed the chicks (Winkler et al. 2013). If many 'cold snaps' occur in one season, it results in high chick mortality regardless of the adults' quality or investment. On the other hand, in good years, when food is abundant, virtually all swallow parents appear able to rear

chicks regardless of variations in quality or investment. Thus, in a species such as the Tree Swallow, chance year effects determine a larger proportion of their fitness, and TL does not have such a big role to play. In comparison, in other species, that are not as dependent on external conditions, TL, as a measure of quality and correlate of potential longevity, might play a bigger role in determining variation in fitness. In this study we included the interaction of year and telomere length as a fixed effect. In all cases year was eliminated as a predictor during model selection. In addition, other studies on aerial insectivores have found TL to predict mortality (Bize et al. 2009; Haussmann et al. 2005; Pauliny et al. 2006), which we do not see here. However, how a species feeds is only one aspect of a multidimensional life-history (Réale et al. 2010). Similarly, year effects could arise from a myriad of causes, from temperature and precipitation to the availability of predators. Singling out the one or a few key traits that affect how TL functions as a metric of fitness, or the key differences between years, will necessitate many more studies on a wider variety of life-histories and taxa.

Lastly, while the predicted direction of the relationship between TL and reproductive success is positive—TL functions as a proxy for quality (Bateson 2016; Le Vaillant et al. 2015)—the individual can potentially respond in such a way to mitigate this effect (Belmaker 2016, chapter 4). If short-telomered individuals increase their investment in current reproduction to compensate for their lower probability of survival (Clutton-Brock 1984; Pianka and Parker 1975; Williams 1966), it could balance out the deleterious effects of short telomeres, at least within one breeding season. There is evidence that individuals with different TL change their behavior (Bateson et al. 2015; Bauch et al. 2013; Belmaker 2016; Ouyang et al. 2016). European Starlings, *Sturnus vulgaris*, suffering higher telomere attrition made more "impulsive" decisions (Bateson et al. 2015). Long-telomered male and female Tree Swallows raised lighter chicks (Ouyang et al. 2016), suggesting they were investing less in their broods. Lastly, in Common Terns, *Sterna hirundo*, individuals

with short telomeres produced more young (Bauch et al. 2013). This interaction of quality and strategic adjustment of investment can produce different pattens of correlation between TL and reproductive success, depending on which is more important in any given scenario. Recent results from our system give some evidence for this and suggest that the strategy employed by Tree Swallows is even more complex then described here, and depends on the TL of the social partner as well as their own (Belmaker 2016, chapter 4).

None of the hypotheses presented above can alone explain all of the patterns observed. However, the important idea here is that the role TL has in determining the fitness of individuals might be context-specific. It might vary as a function of expected lifespan, a particular aspect of the species' life-history or because the individuals adjust their behavior to some degree. It is important to note that TL is not necessarily a causal factor in determining fitness (Simons 2015). Here, we used TL as a marker for a much more complex physiology (Haussmann and Marchetto 2010), and it is very possible that other factors, such as stress or oxidative damage determine fitness as well (Bonier et al. 2009; Haussmann and Marchetto 2010). Despite this, if we replace TL with the term "physiological state" or any equivalent for it, the idea is the same: physiology might determine a different proportion of fitness depending on the particulars of each study. Pinpointing the exact reason why two studies differ is hard to do, but understanding how the context of each study affects the patterns we find will give us a richer view of how TL affects the life history and behavior of species. For this to happen, we need to study more species with a wider range of life histories. In addition, we need to design manipulations and field observations to allow comparisons of the same species across contexts, or comparisons of different species in the same context.

CHAPTER 4

DOES THE RESPONSE TO BROOD ENLARGEMENT OF ADULT TREE SWALLOWS (*Tachycineta bicolor*) VARY WITH THEIR TELOMERE LENGTH?

Abstract

To understand the life histories of organisms it is crucial to study the constraints imposed on an individual's behavior and life history by its physiology. One major component of an individual's fitness is its probability of survival, and this probability has been found to be related to the lengths of an individual's telomeres, which are repetitive DNA-sequences capping and protecting the ends of chromosomes. Shorter telomere length (TL) has been associated with lower probability of survival. Life history theory, specifically the strategic-allocation hypothesis, would predict that individuals with lower probability of survival should be more likely to invest in the current reproductive attempt, compared to individuals with longer TL. To test this hypothesis, we enlarged broods of Tree Swallows (*Tachycineta bicolor*) and predicted that adult responses to this manipulation would vary with their TL. On average, adults in both control and enlarged broods invested the same, regardless of TL. The TL of males and females was significantly correlated, however, and we found surprising evidence for assortative mating for telomere length for the first time in a wild vertebrate. Furthermore, both male and female TL interacted with brood-manipulation group to affect the proportion of chicks fledged, but in opposite ways. Mean wing length of the chicks, the probability of fledging young and adult male return rates were all predicted by a positive interaction of male and female TL. These results do not clearly support the strategic-allocation hypothesis but provide evidence that the strategy employed by individuals depends on the context they confront, including their own quality and their mate's TL. These complicated interactions can ex-

plain the observed variation in the correlation between TL and reproductive success. This study highlights the complexity of the effect TL has on life history and behavior, and it suggests that the TL of the mate should be included in any future predictions of TL effects on life history variation.

Introduction

To fully understand the life history of organisms it is crucial to study the individual (Drent and Daan 1980) and the constraints an individual's physiology impose on its behavior (Monaghan 2014). Physiological constraints can change the costs and benefits of subsequent behavioral decisions, so studying these constraints, and the mechanisms that produce them, can help us understand how behavior evolves and develops (Ricklefs and Wikelski 2002). One major component of an individual's fitness is its ability to survive (Stearns 1992). Factors such as genetic quality, oxidative damage, the stress response and telomere dynamics all interact to affect the individual's probability of survival (Haussmann and Marchetto 2010). Individuals that are not as likely to survive as others (i.e., individuals that are sick or old) can be seen as having lower residual-reproductive-value (RRV), and would benefit from increasing investment in the current breeding attempt at the expense of future opportunities that may not come (Clutton-Brock 1984; Pianka and Parker 1975; Williams 1966). According to this strategic-allocation hypothesis, individuals with relatively low RRV should invest more in the current reproductive attempt to compensate for their higher probability of mortality. Individuals with higher RRV should opt for self-maintenance and investment in future reproduction. While some studies do find this predicted pattern of increased investment with lower RRV (Fischer et al. 2008; Takata et al. 2016; Ward et al. 2009) it could be confounded with other factors, such as aging or experience (Cameron et al. 2000).

Telomeres are long, repetitive sequences capping the ends of eukaryotic chromosomes and maintaining their integrity (Blackburn 2000). Telomeres shorten with each replication as a consequence of the inability of DNA polymerase to synthesize DNA from the 3′ to 5′ direction (Levy et al. 1992). When telomere length (TL) gets too short, the

cell stops dividing and enters a state of replicative senescence (Blackburn 2000), in which the cell does not necessarily die but rather secretes inflammatory cytokines (Rodier et al. 2009), which in turn start a cascade that can lead to reduced function and potentially the death of the individual (Campisi 2005). In addition to their per-replication shortening, telomeres can shorten due to other factors such as oxidative stress (Saretzki and Von Zglinicki 2002) or high activation of the vertebrate stress response (Epel et al. 2004). Because TL is affected by a diversity of both organismal and environmental influences, it can be seen as an integrator of stress (Haussmann and Marchetto 2010). Shorter TL is associated with higher mortality in many species, both in captivity and in the wild, (for a complete list see Haussmann and Marchetto 2010). It is not yet clear if TL has a causal role in the lower probability of survival of short-telomered individuals or if it is just a correlate of mortality rate (Simons 2015). Despite this, because TL does correlate with probability of survival it can be used as a *marker* and a proxy for larger and more complex webs of physiological causation (Haussmann and Marchetto 2010). Because TL can be used as a marker for the probability of survival, short-telomered individuals, on average, have a low RRV while long-telomered individuals have a higher RRV. This means that, based on the strategic-allocation hypothesis, short-telomered individuals should invest more in the current reproductive attempt than should long-telomered individuals. As increased investment in current reproduction is associated with a fast pace-of-life (Réale et al. 2010), if this hypothesis is true, it would mean that TL is associated with the pace-of-life continuum of life history allocations and could help explain variation in many traits simultaneously (Réale et al. 2010; Ricklefs and Wikelski 2002).

To date, there are very few studies to connect TL to strategic decisions for allocation of reproductive effort. An analysis of the reproductive behavior of Tree Swallows, *Tachycineta bicolor*, throughout their life (based on a long-term dataset), found that in their first breeding attempt short-lived females bred earlier and laid more eggs than did long-

lived birds of the same age despite having lower fitness overall (Winkler et al. in prep.). While there was no information about TL in that study, it does show that Tree Swallows might be differentially allocating energy to reproduction based on some unknown cue that predicts their probability of mortality. European Starlings, *Sturnus vulgaris*, suffering higher telomere attrition made more "impulsive" decisions, in agreement with the strategic-allocation hypothesis (Bateson et al. 2015). Long-telomered male and female Tree Swallows raised lighter chicks (Ouyang et al. 2016), suggesting they are investing less in their brood. Lastly, in Common Terns, *Sterna hirundo*, individuals with short telomeres produced more young, but this was interpreted to mean that telomere attrition is a *cost* of high investment (Bauch et al. 2013). However, a different interpretation could be that those short-telomered individuals invested more in reproduction to compensate for their lower probability of survival. It is important to note that these two interpretations are not mutually exclusive. TL can be affected by the life history of an individual on the one hand—the more an individual invests in reproduction the faster its telomeres erode—and TL can affect an individual's life history by changing the costs and benefits of each allocation decision on the other–the strategic-allocation hypothesis. However, this strategic-allocation hypothesis has never been directly tested by measuring both reproductive effort and survival.

While TL correlates with reduced probability of survival it also correlates with reproductive success (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012; but see Lopez-Sarasa 2015), and as such can be used as a proxy for quality as well (Bateson 2016; Bauch et al. 2013; Le Vaillant et al. 2015)—the quality hypothesis. As seen in the previous tern example (Bauch et al. 2013), and pointed out by others (Simons 2015), the direction of causation connecting TL and individual quality is unclear. But TL is associated, perhaps indirectly, with some physiological factor, or combination of factors, that determine the performance of the individual (Haussmann and Marchetto 2010). So when we say TL is a

proxy for quality we mean TL can give us information about the ability of that individual to survive or reproduce—its fitness—regardless whether variation in TL is directly causing variation in these abilities or not. The quality hypothesis and the strategic-allocation hypothesis predict a different pattern of correlation between TL and reproductive success. The quality hypothesis predicts a *positive* relationship between TL and reproductive success, while the strategic-allocation hypothesis predicts a *negative* one. Both a positive (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012), negative (Bauch et al. 2013), and no relationship (Lopez-Sarasa 2015) between TL and reproductive success, have been previously described. Because the predicted patterns for strategy and quality are opposite, the net effect might vary depending on the specifics of the study. Essentially strategy and quality could be masking each other (Bowlin and Winkler 2004; Partridge and Harvey 1988; Smith 1981). However, to our knowledge, no study to date has considered both hypotheses simultaneously in the context of TL variation.

An association between short-telomeres and low survival is a prerequisite to testing the strategic-allocation hypothesis. In Tree Swallows, *Tachycineta bicolor*, TL has been found to shorten with age (Haussmann et al. 2003; but see Belmaker 2016) and has been associated with a reduced probability of survival (Haussmann et al. 2005; but see Belmaker 2016). Haussmann's study was one of the first studies to connect short TL to low survival in a wild bird, and it was conducted in an allied nest-box population of Tree Swallows only 15 km away (Haussmann et al. 2005). As TL has already been shown to correlate with survival in this population, we are encouraged to test the contingent hypothesis that parent Tree Swallows compensate for short telomeres and reduced survival probability by increasing investment in the current reproductive attempt. However, we still know little about how adult Tree Swallows adjust their allocation of reproductive investment in response to physiological cues. To test whether TL in Tree Swallows is associated with a strategic-allocation of investment, and how any strategic-allocation de-

cision interacts with quality, we experimentally enlarged broods of Tree Swallows, while controlling for individual quality and age, and measured several investment metrics. We predicted that the adult's response to this treatment would vary with its TL, such that short-telomered individuals increase investment while long-telomered individuals do not.

Methods

Study system

During the seasons of 2012-2014 we manipulated broods of Tree Swallows, *Tachycineta bicolor*, breeding in Harford, NY. The field site is a cattle-grazing ground where 130 nest boxes are mounted on fence posts ~1.5 m high and spaced at least 20 m apart. The Tree Swallow is a small, migratory aerial insectivore, which is used as a model system in many fields (Jones 2003). Tree Swallows readily breed in man-made nest boxes that facilitate access and measurement. While lone females can at times successfully fledge chicks, both parents are generally needed to complete a breeding attempt (Winkler et al. 2011). Because females might thus be adjusting their own strategy in response to their mate (Pryke and Griffith 2010), we thus also looked at the behavior of males and their TL and related them to the reproductive success of the brood.

Adult capture

Nests were followed daily to detect when the first egg was laid (clutch initiation date) and when the clutch was complete. Females were caught after the seventh day of incubation to minimize the risk of abandonment. Because males do not incubate and do not enter the box until after the chicks hatch, we were not able to catch all males and our male sample sizes are smaller. Females and, as often as possible, males were caught a second time after the chicks were 9 days old. In each capture we measured mass, wing length and head-plus-bill length. In addition, we measured body condition as described in Belmaker (2016, chapter 3). The condition index we used was based on mass, two measures of size-controlled mass, breast-muscle thickness, pectoral score and four fat-store scores. We combined all nine measures into one condition index using a non-linear principle component analysis (Belmaker 2016, chapter 3).

For each individual we also noted its age. Tree Swallow females show delayed plumage maturation, in which second year (SY) birds generally have a brown plumage, and older birds show the characteristic iridescent blue plumage (with 95 % accuracy; Hussell 1983). This allows us to approximate the true age of each female breeder. In addition, for individuals that fledged from our site and returned to breed, we knew their true age with certainty. In cases where no other information was available, we settled for a minimum age of the individual based on its first banding date.

Lastly, a blood sample was taken from the brachial vein for TL analysis. A sample with a minimum of 20 and up to 150 µl was taken with a heparinized micro-capillary tube from the drop of blood resulting from a puncture made in the vein with a 27 gauge needle. The blood was put into an empty 1.5 ml micro-centrifuge tube and stored on ice

until further processing in the lab. At the end of the day, the chilled telomere samples were spun down at 3500 rpm for 5 minutes and the plasma was removed. One ml of NBS buffer (90 % new-born calf serum and 10 % DMSO) was added and mixed with the red blood cells (RBCs). The samples were then frozen slowly and kept at −80°C for storage until analysis.

Experimental manipulation

The experimental manipulation is fully described in Belmaker (2016, chapter 1). In short we paired broods by female age, clutch size and hatch date. Pairing by male traits as well was impossible, as males could not be caught by the time the treatment groups were assigned, if at all. Pairing, by these three traits allows us, as much as possible, to control for the age of the bird, its initial investment and quality. We then swapped half the chicks between each brood and added about ~50 % more chicks to the brood designated as the enlarged one. Final brood sizes for control broods were 5.07 ± 0.67 and 8.05 ± 0.86 for enlarged broods (mean ± sd). Chicks were marked individually by clipping nails, and nest of origin and nest of rearing were recorded for each.

After the swap, nests were visited every four days, each time the chicks being measured again and the clipping renewed as needed to retain chick identification. At the age of 12 days we took a blood sample from the brachial vein of each chick for TL analysis. After that, the chicks were not handled and boxes were only checked briefly and carefully to avoid premature fledging. Once all surviving chicks fledged, the remaining nesting material was scanned to ascertain which and how many chicks died before fledging. The following year we noted which adults returned to breed.

Telomere length analysis

The full procudure for measuring TL is described in Belmaker (2016, chapter 1). In short, we used the TRF method (Kimura et al. 2010). DNA was extracted with a Gentra Purgene extraction kit for the extraction of high quality, high yield DNA (Qiagen, Hilden, Germany), and 10 µg of DNA were digested for at least 16 hours at 37 °C with a combination of three restriction enzymes (RsaI, HaeIII and HinfI). Samples were then frozen until further processing. When ready for processing, samples were quickly thawed at 37 °C and run on a 0.8 % agarose gel in a pulsed-field gel electrophoresis rig for 19 hours (3 V cm^{-1}, 0.5 s initial switch time and 7 s final switch time) alongside three lanes of 1 kb extension ladder from Invitrogen and two standard lanes made of either Domestic Chicken blood or Tree Swallow blood. The gel was then dried and hybridized overnight with a radioactive probe ('CCCTAA' x 4), placed on a phosphor screen (Amersham Bio-sciences, Buckinghamshire, UK) for at least two days and visualized using a Storm 540 Variable Mode Imager (Amersham Biosciences).

Because each cell and each chromosome has a telomere of a different length, this procedure results in smears rather than distinct bands, each smear representing the distribution of telomere lengths for each individual (Kimura et al. 2010). From each telomere distribution we measured mean TL, skew, kurtosis and the 10th to 90th deciles. We then combined all these measures into one metric for TL, as described in Belmaker (2016, chapter 1).

Measuring investment

We hypothesized that individuals with short telomeres would have higher reproductive investment and, when experimentally challenged, would increase investment in the current reproductive attempt to compensate for their lower probability of survival. We measured the investment of each individual by using several metrics. First, the decline in body condition between the two captures, as measured by our condition index (see above). Birds that work harder should show a steeper decline in body condition as their energy goes into rearing chicks rather than self-maintenance. In our analyses a higher decline in condition corresponds to a more positive number. We controlled for the natural decline in condition throughout the season (Boyle et al. 2012) by using the residuals of a regression of condition on days before hatching, sex and capture number (first or second). Second, the reproductive success of each brood: birds that work harder should fledge more young, either in absolute terms or as a proportion of the number of chicks in the brood. Third, the size of chicks at day 12: chicks that were better taken care of should have grown to be larger on day 12. Lastly, the adult's probability of return: as survival trades off with reproduction, a higher investment in reproduction should translate to lower return rates. While return rates confound dispersal with true survival they are commonly used as a proxy for survival (e.g., Angelier et al. 2013). In NY Tree Swallows in particular, breeding dispersal is low (Winkler et al. 2004) so return rates closely approximate true survival. While it might seem that our definition of investment is circular—our metrics for investment are the same as the consequence of increased investment—this is not the case, as we are using the *difference* between the treatment groups as an indication of increased investment not the metrics per se (see below).

Predictions

This study challenged adult Tree Swallows by adding chicks to a brood. This increase in demand gives adults the option to invest more and fledge a greater number of chicks, or invest as they would have and risk fledging fewer young that would be in lower condition. Given the effect of TL on survival, short-telomered females are expected to increase investment more in response to the brood enlargement than would females with longer telomeres, as they have much less to gain by withholding effort (Clutton-Brock 1984; Pianka and Parker 1975; Williams 1966). This would predict that the response to brood enlargement will vary with TL: The body condition of short-telomered females is expected to decline more steeply than should that of long-telomered females; the chicks of short-telomered females are expected to grow bigger than those of long-telomered females; short-telomered females with enlarged broods are expected to fledge more young compared to long-telomered females; and short-telomered birds are expected to suffer higher mortality.

It is important to note that in all cases we expect the *difference* between enlarged and control broods to vary with TL. This is because we are using our control birds as the baseline measure of investment for an individual of a given age that laid that many eggs on a given date. The response to the treatment is the amount the adult with an enlarged brood *differed* from that baseline. In reality, actually pairing the data points by subtracting the value of the control brood from that of the enlarged one creates two complications. First, because experimental groups had to be assigned before TL could be measured, females could not be paired by TL. Second, when looking at probabilities like return rates it is hard to do this subtraction, as the values are either one for a bird that returned and zero for one that did not. The solution is to look at the *predicted* values of each measure given a bird's TL in the control brood and compare that to the predicted value for the enlarged

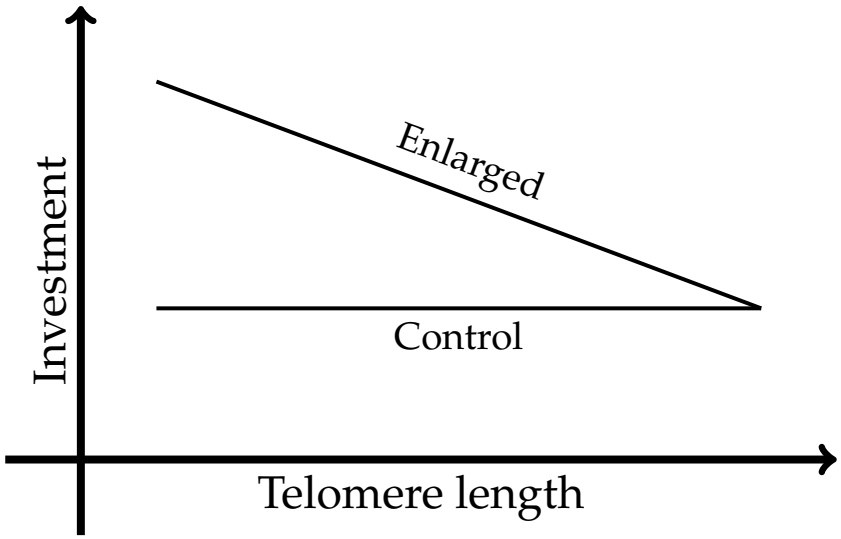

Figure 4.1: A schematic plot representing the predicted results under the strategic-allocation hypothesis. According to this hypothesis long-telomered individuals with enlarged broods will invest the same energy in the current reproductive attempt (y axis) as a comparable individual with a control brood. In contrast, short-telomered birds in enlarged broods will invest more in the current reproductive attempt than a comparable bird with a control brood, as they have nothing to gain by withholding investment. The predicted line for control broods is drawn here as flat, but the actual slope might vary based on other factors, such as the quality of the individual (Bauch et al. 2013; Le Vaillant et al. 2015). The important point of this plot is to see how the difference between control and enlarged broods varies with TL.

one. This results in a model with our measure of investment as the response variable and the interaction of TL and experimental group as our fixed effect. If this interaction term is significant we can deduce that the response to the treatment varies with TL (Fig. 4.1).

The possible compensating effect of male effort

This experiment was designed with females in mind, as they are easier to catch and manipulate. However, males contribute a potentially large portion of reproductive effort, and females too, may vary their investment according to their perception of potential

male contributions to care (Pryke and Griffith 2010). Even though our sample sizes are larger for females than for males (n = 78 and n = 67 respectively), we were able to look at such interaction effects. First, we looked at assortative mating with respect to TL and other traits. Second, we tested each sex individually by adding the interaction of either male or female TL with experimental group. Lastly, we tested whether male and female TL interactively affected nesting success by adding the interaction term between them to our models.

Statistical data analysis

For all analyses we used R (version 3.0.2; R Core Team 2015). Mixed models were done using the 'lme4' package (version 1.1-11; Bates et al. 2013) with functions 'lmer' and 'glmer'. Plots of the data show that in all cases linear models are appropriate so we did not separately test non-linear effects of TL. Some birds were manipulated more than once across years (mainly males, as they were only caught after treatment group was assigned). However, when bird 'ID' was added as a random effect a plot of the residuals over fitted values showed a clear pattern indicating that not all data points were independent. To solve this problem, the first duplicate record was kept, and all other records with the same male or female were removed. This left 56 records in our dataset, half control and half enlarged broods. The resulting diagnostic plots showed no violation of model assumptions. Year was added as a random effect in all models.

The main effect we wanted to test is the interaction of adult TL and experimental group. To test the response to the manipulation of each sex separately we added the interaction of male TL and experimental group and female TL and experimental group

to the model. We tested how male and female TL jointly affect our response variables by adding the interaction of male and female TL as a predictor. The final fixed effect structure we used was in the form of: Male TL *x* Experimental group + Female TL *x* Experimental group + Male TL *x* Female TL. An alternative third-order interaction, with male and female TL and experimental group, was tested and found to be non-significant for all models except for male return rates. To test for assortative-mating we used a separate linear mixed-model with male trait value as the response, the female's trait value as a predictor and year as a random effect.

In all analyses, count data, such as number of chicks fledged, were fit with a Poisson family and proportional data and presence/absence type data, such as probability of survival, were fit using the binomial family. Nesting success was estimated by the probability of fledging at least one chick, the absolute number of chicks fledged and the proportion of chicks fledged. For the latter two, only nests that fledged at least one chick were included in the analysis. The full model was then simplified through a stepwise selection process, where each term was dropped sequentially and testing the AIC value of each sub-model. The dropped term that most improved the AIC value was then taken out. We then tested the addition of previously removed terms to make sure none were removed erroneously. The final model was reached when no deletion or addition of terms could further improve the AIC. P-values for components of the final model were calculated using likelihood ratio tests.

The focus of this study is to test the strategic-allocation hypothesis. While testing the differences between years is interesting and worthwhile, the low sample size, model complexity and unbalanced nature of the data make it difficult to properly model these effects. In addition, there are many potential random differences between years. Trying to isolate the exact cause for each year effect we find will detract from the overall goal of

the study to test reproductive investment. We therefor chose to include year as a random factor rather than a fixed effect.

In a few cases the interaction of male and female TL was kept in the final model. However, as both are continuous variables, plotting this interaction is difficult. In these cases we chose to plot the fitted regression line accompanied by the point-wise 95 % confidence bands. This method precludes us from adding the data points, as the plotted trendline does not match the points. We feel that this method, despite omitting the data points, gives the reader the best summary of the reported effect.

Results

In total, 39 paired brood manipulations were conducted: 16 in 2012, 9 in 2013 and 14 in 2014. After removing cases where the same individual was manipulated more than once, we were left with 56 breeding pairs: 27 2012, 6 in 2013 and 23 in 2014. As we are not pairing the controls and manipulated pairs statistically (see methods section) this leaves 25 control pairs and 31 enlarged pairs. There was no difference in the TL of birds with enlarged broods and those with control broods (Welch two sample t-test; males: $n_1 = 29$, $n_2 = 25$, $t_{48.60} = -0.14$, $p = 0.89$; females: $n_1 = 31$, $n_2 = 25$, $t_{41.95} = -0.70$, $p = 0.49$). The TL of neither males nor females changed with age, so there was no need to control for age in our models (males: $n = 54$, $\beta = 0.02 \pm 0.48$, $t_{52} = 0.05$, $p = 0.96$; females: $n = 56$, $\beta = 0.16 \pm 0.47$, $t_{53.76} = 0.35$, $p = 0.73$). This was true when using minimum age as well as the known age of each bird.

Assortative mating

Male and female TL were positively correlated ($n = 54, \beta = 0.32 \pm 0.14, \chi^2_1 = 5.28, p = 0.02$; Fig. 4.2) as were their wing lengths ($n = 55, \beta = 0.37 \pm 0.15, \chi^2_1 = 6.07, p = 0.01$). Male and female head-plus-bill length, minimum age, and condition were not significantly correlated (Head-plus-bill: $n = 55, \beta = 0.02 \pm 0.16, \chi^2_1 = 0.05, p = 0.82$; Minimum age: $n = 55, \beta = 0.15 \pm 0.14, \chi^2_1 = 1.08, p = 0.3$; Condition: $n = 30, \beta = 5.11 \times 10^{-2} \pm 1.94 \times 10^{-1}$, $\chi^2_1 = 0.02, p = 0.88$).

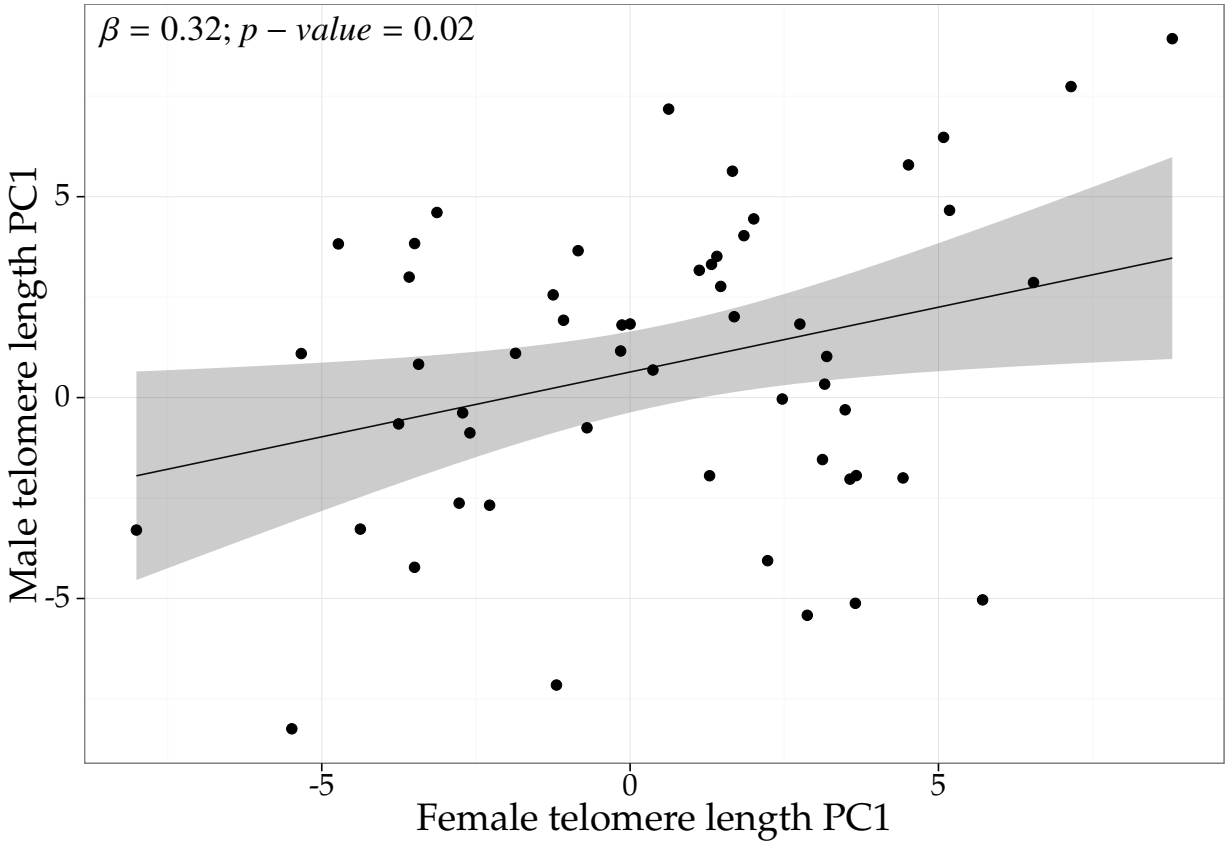

Figure 4.2: The correlation of the telomere length of the male and female of each pair of Tree Swallows measured. The line plotted is the fitted regression line, and the gray zone represents the 95 % point-wise confidence bands.

Nestling size

The brood enlargement significantly retarded chick growth and decreased fledging probability. The mean mass, wing length and head-plus-bill length of chicks in enlarged broods was smaller than in control broods (mean mass: $n = 48, \beta = -2.6 \pm 0.58, \chi^2_1 = 16.99, p < 0.001$; wing length: $n = 48, \beta = -8.29 \pm 2.04, \chi^2_1 = 15.64, p < 0.001$; head-plus-bill length: $n = 48, \beta = -0.80 \pm 0.26, \chi^2_1 = 9.01, p = 0.003$). The individual chick's probability of fledging was significantly lower in enlarged broods (Belmaker 2016, chapter 1).

Male TL, female TL and their interaction with experimental group were not retained as a result of the model selection process for the models for mean chick mass and mean chick head-plus-bill length ($p > 0.22$ for all cases). The interaction of male and female TL was retained in the final model for mean chick wing length based on AIC, but was borderline significant based on the likelihood ratio test ($\beta = 0.1 \pm 0.06, \chi^2_1 = 2.71, p = 0.1$; Fig. 4.3). The coefficient for this interaction is positive, which means that a unit increase in the male's telomeres makes the relationship between female TL and mean chick wing length more positive.

Nesting success

The probability of fledging any young was predicted by an interaction of male and female TL ($\beta = 0.08 \pm 0.04, \chi^2_1 = 5.38, p = 0.02$; Fig. 4.4), such that when paired with a short-telomered mate the probability of fledging young goes down with female TL and goes up with female TL when paired with a long-telomered mate. Experimental group was not an important predictor of the probability of fledging at least one young either in isolation

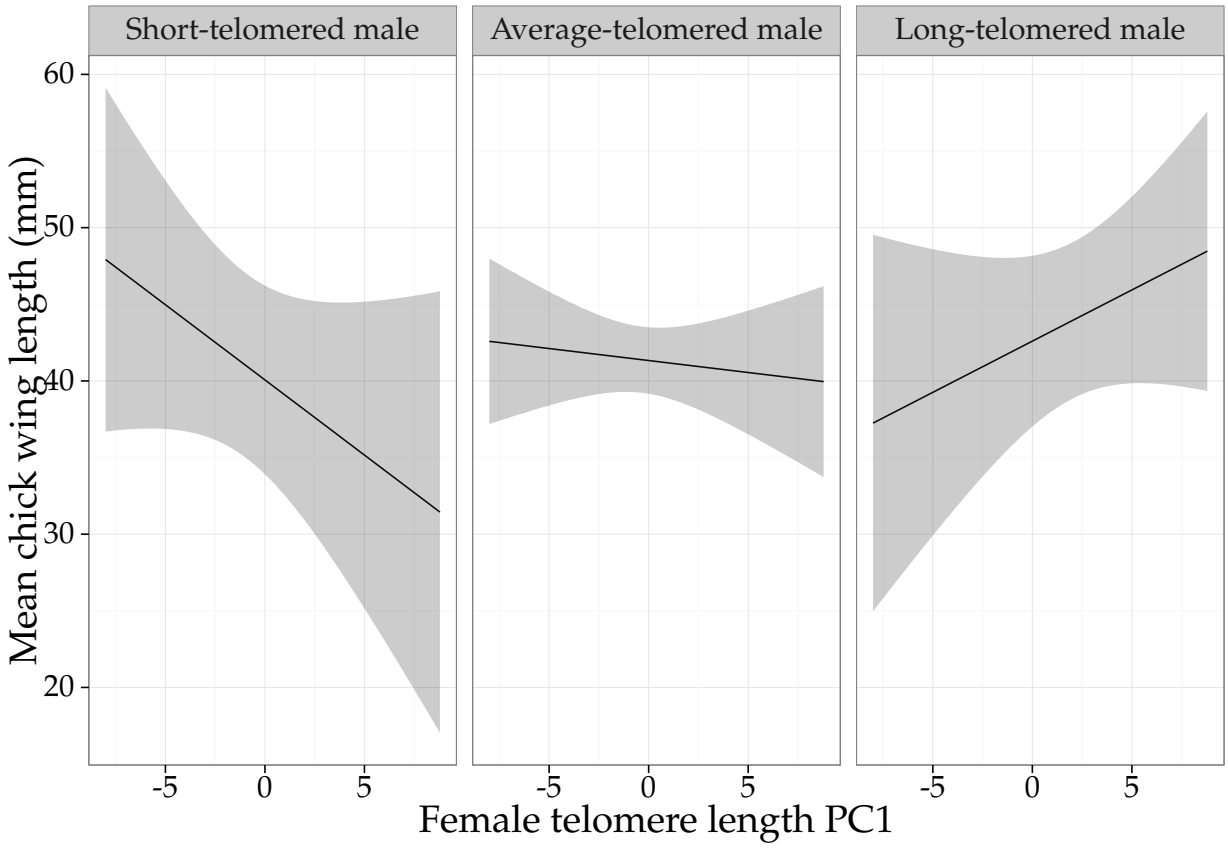

Figure 4.3: An effect plot of the interaction effect of male and female TL on mean chick wing length at day 12. The plot shows the effect of female TL on the mean wing length of 12-day-old chicks when the mother is paired to a short-telomered male (minimum male TL, left), average-telomered male (average male TL, center) and long-telomered male (maximum male TL, right). The plot here shows the effects and the 95 % confidence bands but, as both male and female TL are continuous measures, we could not plot the actual data points.

($\beta = -0.94 \pm 0.78, \chi^2_1 = 1.54, p = 0.21$) or interacting with female TL ($\beta = 0.17 \pm 0.34, \chi^2_1 = 26$, $p = 0.61$) or male TL ($\beta = -0.33 \pm 0.24, \chi^2_1 = 1.96, p = 0.16$).

The proportion of chicks each successful pair fledged was reduced in enlarged broods ($\beta = -2.9 \pm 0.62, z = -4.633, p < 0.001$; Fig. 4.5), but, because enlarged broods artificially contained additional chicks, this did not result in an effect of experimental group on the absolute number of chicks fledged for the pairs that produced at least one fledgling

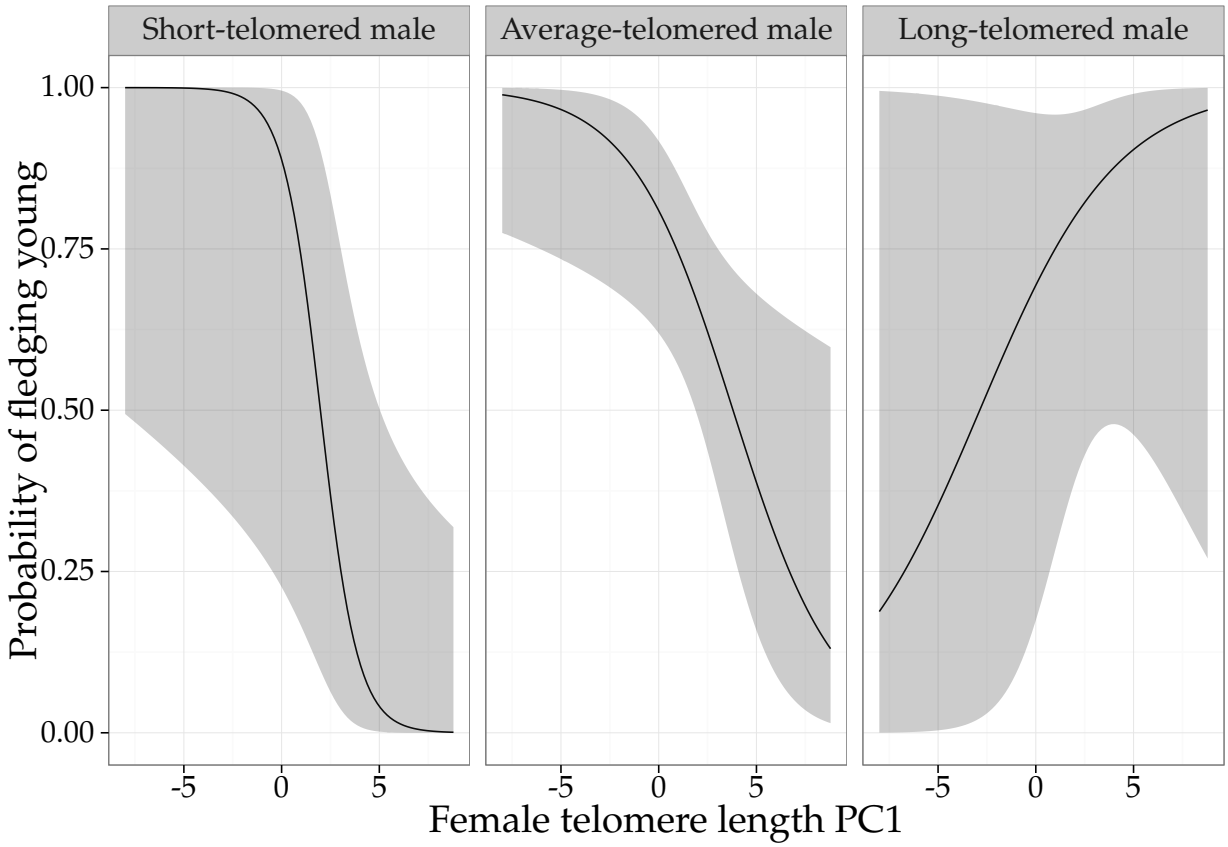

Figure 4.4: An effect plot of the interaction effect of male and female TL on the probability of fledging at least one chick. The plot shows the effect of female TL on the probability of fledging at least one chick when the mother is paired to a short-telomered male (minimum male TL, left), average-telomered male (average male TL, center) and long-telomered male (maximum male TL, right). The plot here shows the effects and the 95 % confidence bands but, as both male and female TL are continuous measures, we could not plot the actual data points.

($\beta = -0.04 \pm 0.15$, $\chi^2_1 = 0.09$, $p = 0.76$). Both male TL and female TL interacted with experimental group to affect the proportion of chicks fledged but in opposite ways: in control broods female TL was *positively* associated with the proportion of chicks fledged ($\beta = 0.29 \pm 0.12$, $\chi^2_1 = 4.76$, $p = 0.03$; Fig. 4.5A, blue line) while male TL was *negatively* associated with it ($\beta = -0.29 \pm 0.16$, $\chi^2_1 = 3.63$, $p = 0.05$; Fig. 4.5B, blue line). In enlarged broods, neither the female nor male TL effect were significantly different than zero (females: $\beta = 0.03 \pm 0.05$, $\chi^2_1 = 0.56$, $p = 0.45$; Fig. 4.5A, orange line; males: $\beta = 4.3 \times 10^{-4} \pm 0.04$,

$\chi^2_1 = 9.95 \times 10^{-5}$, $p = 0.99$; Fig. 4.5B, orange line). When we analyze the proportion of chicks fledged by each parental sex separately, we do not find the predicted interaction between TL and experimental group (females: $\beta = -0.11 \pm 0.10$, $\chi^2_1 = 1.38$, $p = 0.24$; males: $\beta = 0.06 \pm 0.10$, $\chi^2_1 = 0.33$, $p = 0.56$)

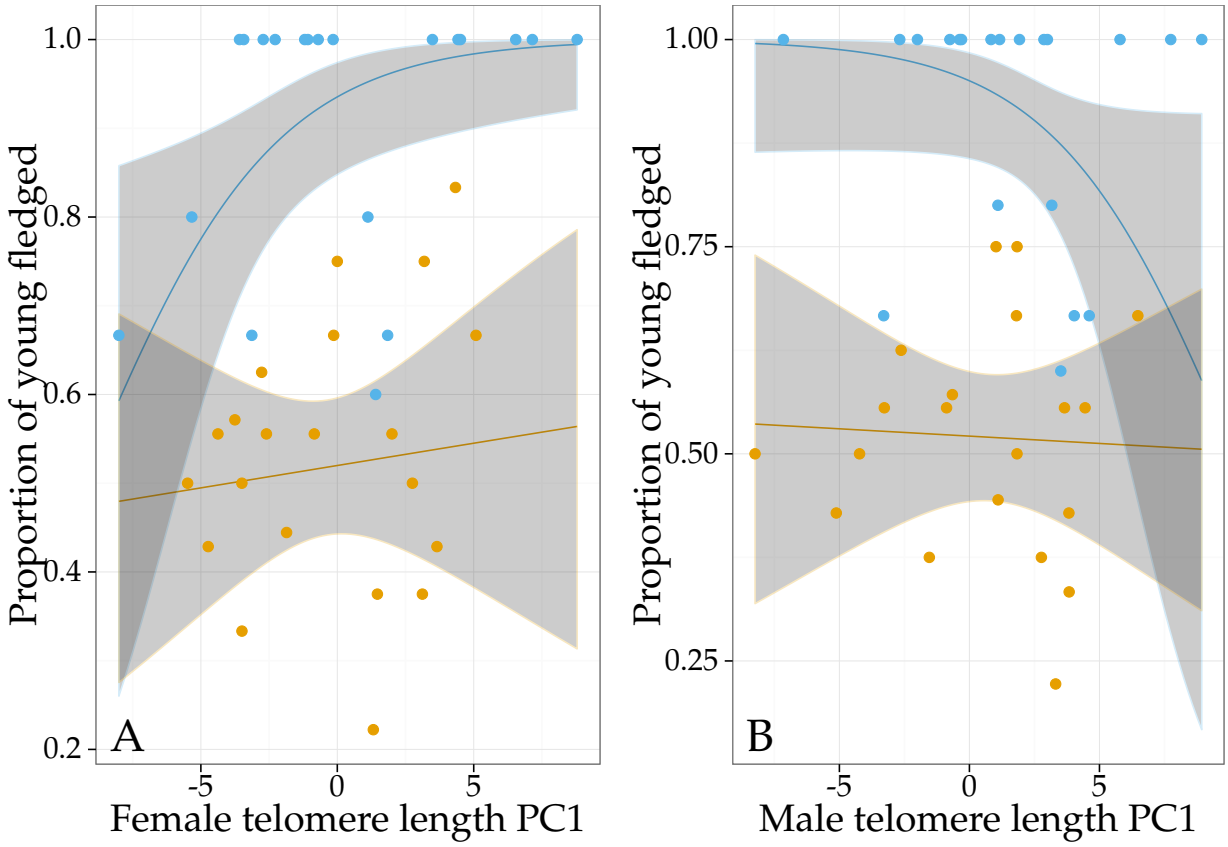

Figure 4.5: The proportion of young fledged in broods fledging at least one chick as a function of female (A) and male (B) telomere length. Blue lines are the predicted regression line for control broods and orange lines are the predicted lines for enlarged broods. Gray zones are the point-wise 95 % confidence bands.

Decline in condition

The decline in condition between captures of the *female* was predicted by an interaction of *male* TL and experimental group such that in control broods females mated to long-telomered individuals declined less in condition than did females mated with short-telomered males, while in enlarged broods this trend was reversed (The effect of male TL in control broods: $\beta = -7.0 \times 10^{-4} \pm 5.0 \times 10^{-4}$; The effect of experimental group: $\beta = 9.7 \times 10^{-5} \pm 2.5 \times 10^{-3}$; The interaction effect of male TL and experimental group: $\beta = 1.9 \times 10^{-3} \pm 6.7 \times 10^{-4}$, $\chi^2_1 = 7.46$, $p = 0.006$; Fig. 4.6). The best model for the male decline in condition only included an intercept term.

Return rates

Female TL predicted female return rates but the relationship was negative and only borderline significant despite being retained in the final model based on AIC ($\beta = -0.15 \pm 0.08$, $\chi^2_1 = 3.37$, $p = 0.07$). On the first round of model simplification the model for male return rates retained the third-order interaction of male TL, female TL and experimental group ($\beta = 0.20 \pm 0.11$, $\chi^2_1 = 6.47$, $p = 0.01$). The model revealed that in all regressions in control broods there was no relationship between male TL and return rates, regardless of female TL. We thus further simplified the model by running it again only in enlarged broods. The best model for the enlarged broods alone included the interaction of male and female TL, such that for females mated to short-telomered males the relationship between female TL and return rates is negative and the relationship become more and more positive when mated with longer-telomered males ($\beta = 0.18 \pm 0.09$, $\chi^2_1 = 8.59$, $p = 0.003$; Fig. 4.7).

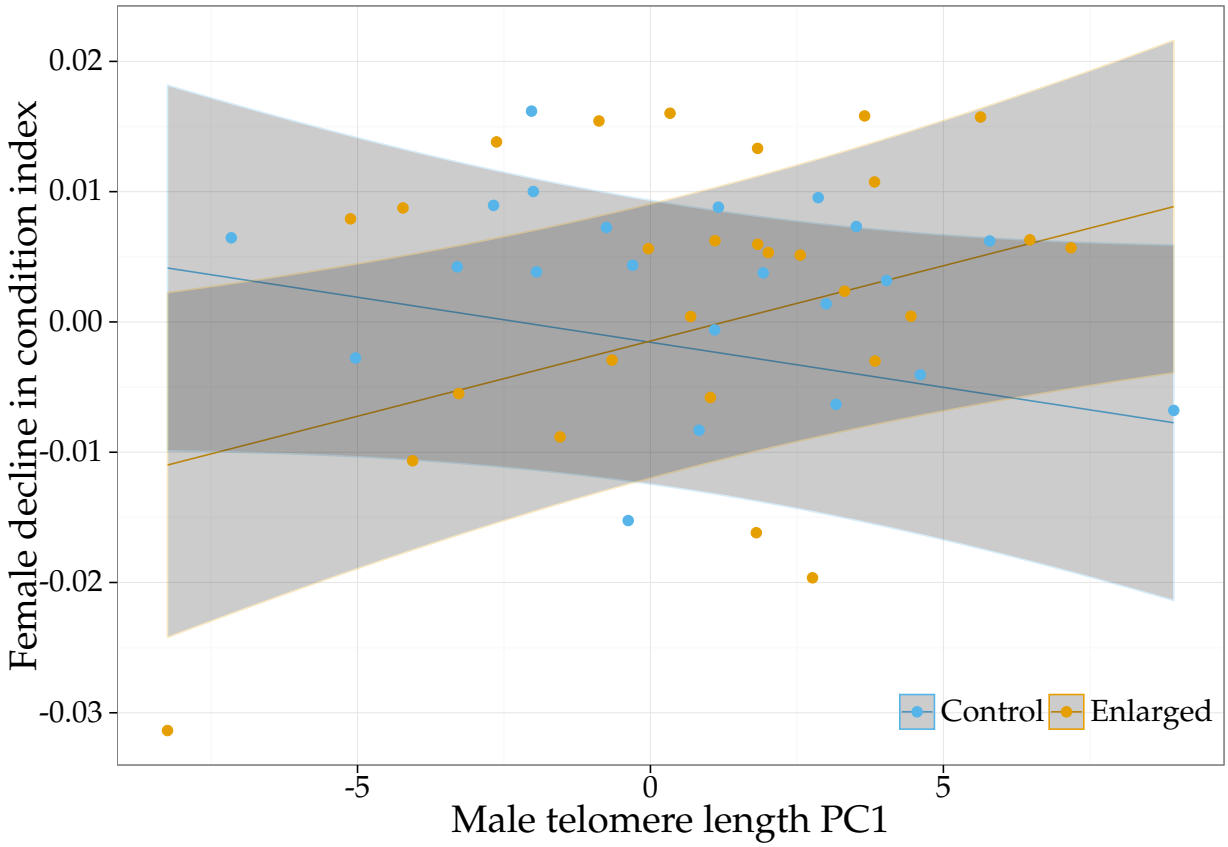

Figure 4.6: The decline in condition of *females* as a function of *male* telomere length, in control (blue) and enlarged (orange) broods. A higher number on the y-axis signifies a steeper decline in condition. The extreme datum on the bottom left is 3.4 standard deviations lower from the mean. When it is removed the interaction is still an important predictor of female decline in condition based on AIC but is no longer significant at the 5 % level based on likelihood ratio tests. Both lines are the fitted regression lines and the gray zones are the point-wise 95 % confidence bands.

Discussion

This study tested the hypothesis that adult Tree Swallows compensate for a low probability of survival (i.e., short TL) by increasing investment in the current reproductive attempt. A negative interaction effect between adult TL and experimental group on any of our measures of investment (decline in condition, fledging success, chick size and return rates) would provide evidence in favor of the strategic allocation hypothesis (Fig. 4.1).

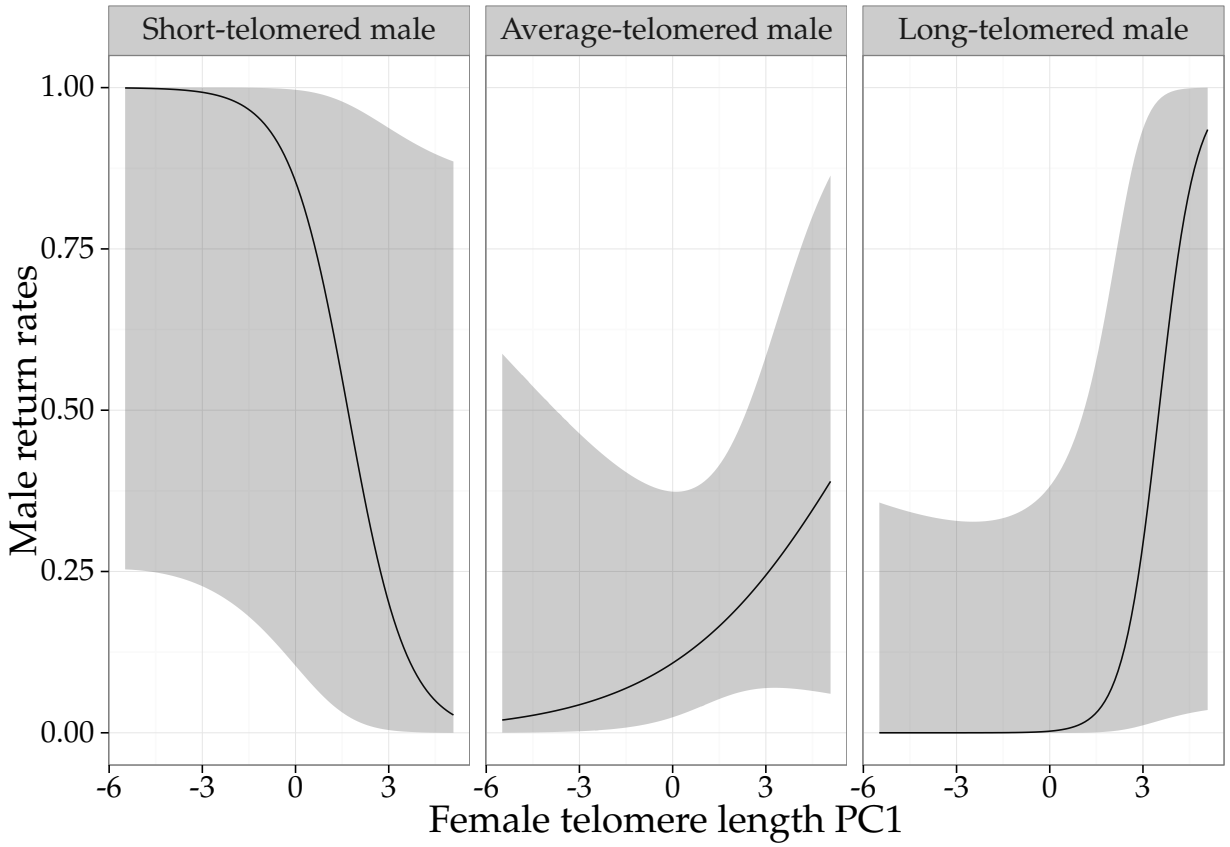

Figure 4.7: Plot of the interaction effect of male and female TL on the return rates of males attending enlarged broods. The plot shows the effect of female TL on her return rate when the mother is paired to a short-telomered male (minimum TL male, left), average-telomered male (average TL male, center) and long-telomered male (maximum TL male, right). The plot here shows the effects and the point-wise 95 % confidence bands but, as both male and female TL are continuous we could not plot the actual data points.

The brood enlargement was successful in creating a challenge for adult birds—chicks in enlarged broods were smaller on day 12 and suffered greater mortality. However, at first glance, it does not seem that the manipulation had much of an effect on the behavior of adults. Experimental group, as a main effect, failed to be included in any of the final models except chick size. The lack of effect of experimental group means that, on average, adults with enlarged broods lost as much condition, had the same probability of fledging young and fledged the same number of chicks, as controls. The proportion of chicks fledged was lower in enlarged broods but only because they artificially contained extra

chicks. The predicted interaction between TL and experimental group was found only for the proportion of young fledged but for males and females in opposite directions.

Although other studies have found a correlation between TL and survival (Angelier et al. 2013; Bize et al. 2009; Haussmann et al. 2005; Heidinger et al. 2012; Salomons et al. 2009; Stier et al. 2014; Verhulst et al. 2006), recent results from Tree Swallows show that TL might not be as important in Tree Swallow reproductive behavior as it is in other species (Belmaker 2016, chapter 3). In our system, TL did not correlate with return rates (Belmaker 2016, chapter 3), and the strategic-allocation hypothesis depends on a positive relationship between TL and the probability of survival. Here, although the correlation is not very robust, what relationship we see is a *negative* relationship between TL and return rates in females and an interaction effect for males. If TL does not predict the probability of survival it could explain why, on average, we do not see an interaction between TL and experimental group, as predicted.

Another possible reason for the apparent failure of the strategic-allocation hypothesis is the way we paired the control and enlarged broods. This experimental design uses the control broods as a baseline to measure the response to the treatment of the adults raising enlarged broods. For this to be an accurate comparison, both control and enlarged broods need to be "clones" for all practical purposes. However, despite the fact that control and enlarged broods were paired by female age, hatch date and clutch size, the paired birds could clearly differ in many other key traits. Most importantly, the control and experimental broods were not matched for female TL. Because we could not measure TL until after the experiment was performed, the control female in each paired brood cannot be used directly as a baseline for the experimental female's behavior. TL has been previously used as a proxy for individual quality (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al. 2012). As we could not pair females by TL, variation in individual quality, as

indicated by TL, might be confounding our results. However, we did *not* pair control and enlarged broods statistically (see methods) and on average there was no difference in TL between adults with control or enlarged broods. In addition, as stated before, in our system, on average, female TL did not correlate with reproductive success (Belmaker 2016, chapter 3). Taken together, this means that the fact that we did not pair females by TL is not sufficient to explain the apparent lack of a strategic-allocation effect.

While quality differences between birds with control and enlarged broods could not fully explain our results, quality can interact with strategic-allocation in ways that can mask its effects. First, the strategic-allocation hypothesis predicts a negative correlation between TL and annual reproductive success while the quality hypothesis predicts a positive one. If both effects are equally strong they could balance each other out and on average we would not see any correlation between TL and reproductive output, as in Belmaker (2016, chapter 3). Second, If short-telomered birds are lower quality they might be maxed-out and could not increase investment, even if it beneficial to do so. In this case we will not see an interaction between experimental group and TL. So while, on average, these results do not support either the quality or the strategy hypothesis directly, the two can interact in unexpected and complex ways that will make it harder for us to detect their effects.

The potential way strategy and quality interact to affect reproductive investment if further complicated when we consider the role males play in the Tree Swallow breeding cycle. Control and experimental groups were not paired by any male characteristic. However, males are an integral part of nesting success in Tree Swallows—they help defend the nest and feed the young (Winkler et al. 2011)—and they are likely an important part of any strategic decision a female makes (Pryke and Griffith 2010). This failure to account for any male characteristics in the pairings for the experiments grows especially interest-

ing and potentially important when we consider the surprising evidence for interactions between the TL measures of the mates in this study. First, we found that the male and female of a pair have similar TL (Fig. 4.2). Only one other study has found a correlation between the TL measures of a couple, and that was conducted in humans (Broer et al. 2013). However, Broer et al. (2013) interpreted this to mean that the shared environment the couple lived in induced the similarity in TL. In Tree Swallows, pairs often change in successive seasons, and they seem unlikely to share the same environment off the breeding ground (Laughlin et al. 2016), so this explanation does not seem relevant here. It seems much more likely that birds are responding to something during mate choice that reflects the TL of the mate. While individuals are probably not choosing TL directly, they might be choosing a healthy mate and TL correlates with good health (Bateson 2016), resulting in the assortative mating patterns we see here. Alternatively, birds with different TL might have very slightly different behavior, differences in reaction times or intensities that may well be all but invisible to humans but very important to individual swallows during courtship and mate choice. Some birds might not be able to avoid "choosing" a short-telomered mate if all the long-telomered individuals are already paired. Alternatively, there might be an advantage to choosing a short-telomered mate depending on your own TL (see below).

Second, the proportion of chicks fledged in control broods shows associations with TL that are positive in females and negative in males (Fig. 4.5). One possible interpretation is that with females individual quality plays a bigger role in determining the proportion of chicks fledged than strategy—we see a positive correlation. In contrast, males use strategy and are less cued in to their quality—we see a negative association between TL and the proportion of young fledged. While trying to explain such patterns is tempting, we need to interpret them with caution. Adults with enlarged broods did not show similar patterns, and we do not see this mirror-image pattern in the effects of TL on

chick size, declines in condition or return rates. One would think that if this pattern were general we would see it manifested with other metrics of investment as well. Regardless of the exact reason behind this pattern, it does highlight the fact that males and females might be responding differently to the same environment and/or the same physiological state.

The assortative mating and the sex-dependent effect on the proportion of chicks fledged suggest that the interaction of males and females may be more complex than we predicted. There are three lines of evidence to support the idea that a female's individual strategic-allocation is dependent on the TL of the male. First, the decline in condition of *females* is dependent on the TL of *males* but not on their own TL. Second, when we analyze the proportion of chicks fledged with each sex separately we do not find the interactions between TL and experimental group. We do, however, find them when including the TL of the mate in the model (Fig. 4.5). Lastly, the final models for both the probability of fledging at least one chick and of mean chick wing length included the interaction term of male and female TL with a positive coefficient. The interaction of male and female TL also comes out as an important predictor of male return rates, but only for males attending enlarged broods. The interaction effect of male TL and female TL was initially included in all models and after the model selection procedure failed to be retained in most models we examined. However, even though the male-female TL interaction was only retained in three cases, the initial coefficient estimate for this interaction in eight out of ten models was positive. These positive interaction coefficients indicate that an increase in the male's TL results in an increase in the slope of the regression between the female's TL and the response variable. The fact that in most of these cases the coefficient has the same sign means that, in whatever metric we look at, the effect of male TL on the behavior of the female is consistent. The one response variable that is marked by negative male-female TL interactions is the decline in condition. It is possible that the decline in condition is

different because it might not measure strategy as much as we initially assumed. There is evidence that Tree Swallows lose fat mainly after chick hatching to be more efficient fliers (Boyle et al. 2012). Thus, the loss of mass and fat might not measure the level of investment in current reproduction. This might explain why TL does not predict the decline in condition in this study—while TL does predict this body condition index at the beginning of the season (during incubation, Belmaker 2016, chapter 3), the *change* in this index throughout the season is not related to TL, but rather to flight efficiency (Boyle et al. 2012).

The general pattern that emerges from the interaction of male and female TL is that the slope of the regression on TL is negative when paired to a short-telomered mate, flat when paired to an average mate and positive when paired to a long-telomered mate (Figs. 4.3, 4.4, and 4.7). In the results section we plotted female TL on the x-axis and divided male TL into three. This was done for illustration purposes only and we could have plotted male TL on the x-axis instead. The idea is that both female and males dynamically respond to their own state, their mate's and to the environment. If TL is really a good proxy for quality one would predict a positive relationship between TL and reproductive success. On the other hand, if the strategic-allocation hypothesis is true one would predict a *negative* correlation between TL and annual reproductive effort. The interaction of male and female TL suggests that both could be coming into play, depending on the mate—when mated to a short-telomered individual we see the negative pattern consistent with strategic allocation, and when mated to a long-telomered individual we see the pattern suggestive of quality effects (Figs. 4.3, 4.4, and 4.7). This suggests that, when studying the association between TL and reproductive success, we might get different patterns unless we control for the mate. Thus, short-telomered individuals viewed independently of their mates might be seen as performing better than long-telomered individuals (Bauch et al. 2013), worse (Le Vaillant et al. 2015; Pauliny et al. 2006; Plot et al.

2012) or equally well (Belmaker 2016; Lopez-Sarasa 2015). This pattern also suggests that to achieve high annual reproductive success either both members of a pair need to be of higher quality, managing to provide adequate care to the young without large reproductive effort, or both need to be short-telomered, strategically allocating larger reproductive effort to provide sufficient energy to the current reproductive attempt. It seems possible that matching strategies in both mates could yield higher reproductive success than in pairs with one of each strategic type. This means that a short-telomered individual might be better off pairing with another short-telomered individual rather than choosing a higher-quality long-telomered mate. The assortative-mating pattern we found strengthens this idea.

These results taken together suggest that interactions between the mates, even in short-lived organisms with large environmental variability, can influence many aspects of reproductive effort. The mechanisms of how interactions between parents could produce the correlations in effort and success documented here are a fascinating avenue for future detailed research. But it is clear that the strategies and constraints in play between interacting avian parents are not necessarily simple or constant, even within a single reproductive season. Patterns such as these warrant further investigation, and we hope they inspire more studies into the ability of adults to respond adaptively to their physiological state, their mate and the environment.

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